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ASEXUAL REPRODUCTION OF *PLASMODIUM KNOWLESI* IN RHESUS MONKEYS

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Changes which occur during the course of malarial infections in the rate of merozoite production and in the death and survival of the progeny so formed have been shown to be fundamental in evaluating the plasmodicidal and reproduction-inhibiting effects in the antimalarial action of immunity (L. G. Taliaferro, 1925; Boyd, 1939; W. H. and L. G. Taliaferro, 1944 and 1947), of quinine (original work and review in W. H. and L. G. Taliaferro, 1949), and of immunity as modified by nitrogen mustard (W. H. and L. G. Taliaferro, 1948). In addition, it is highly desirable to obtain such data in a number of species of malaria because the evidence to date indicates the occurrence of striking differences among various species.

This paper presents detailed data for blood-induced infections of *Plasmodium knowlesi* in rhesus monkeys and has been reported in a preliminary form by W. H. Taliaferro (1948a). It gives data on the length of the asexual cycle and the number of merozoites formed by parasites during each asexual cycle, which together give the rate of reproduction of the synchronously reproducing erythrocytic stages of plasmodia causing many malarias. In addition, it gives parasite counts, from which, in conjunction with the rate of reproduction, can be computed survival and death rates of the parasites formed at each segmentation.

The data are discussed with respect to the effect of the host's innate and acquired immunity on the parasite as has previously been done for *P. brasilianum* in Central American monkeys (W. H. and L. G. Taliaferro, 1944), for *P. floridense* in lizards (Thompson, 1944) and for *P. cynomolgi* in rhesus monkeys (W. H. and L. G. Taliaferro, 1947). Many of these investigations grew out of work on *P. cathemerium* by the junior author (1925), who first drew attention to the possibility of obtaining rates of reproduction in the plasmodia and studied the length of the asexual cycle and the over-all death of parasites during the course of infection; by Hartman (1927), who separated extra- and intracorpuscular death of erythrocytic stages of *P. cathemerium* during the acute rise and especially studied intracorpuscular death; and by Boyd (1929a and b), Boyd and Allen (1934) and Boyd (1939), who first adequately emphasized changes in the merozoite mean per segmenter during the course of infection.

Prior to the present study on *P. knowlesi*, the following relevant data have accumulated on infections with *P. knowlesi*. The morphology and synchronism of *P. knowlesi* and its lethal effects have been amply reported by Sinton and Mulligan (1933), Mulligan and Sinton (1933), Brug (1934), Mulligan

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We wish to thank Mrs. Jean Launspach Smith for technical assistance.

(1935), Coggeshall (1937), Coggeshall and Kumm (1937 and 1938) and Coggeshall and Eaton (1938). Brug (1934) reported that increases during the patent period were several times higher than could be explained by the average value of 9 merozoites per segmenter. He explained this finding by assuming that parasites reproduced in the tissues and were periodically released into the peripheral blood. Afridi (1938) verified the sudden increases in parasitemia, but did not find large numbers of parasites stored in the spleen prior to the sudden rise in the peripheral blood. He also noted irregularities in the asexual cycle and the appearance of degenerate segmenters similar to what we term crisis forms.

MATERIALS AND METHODS

One strain of *P. knowlesi* was used which was furnished us by Dr. R. J. Porter in January 1944 and is the strain previously used by Coggeshall (1937). All infections were blood-induced in the rhesus monkey, *Macaca mulatta* (=*Silenus rhesus*).

Most of the procedures used have already been described by us (1947). Consequently, only a few important or different ones will be briefly mentioned here.

Monkeys were fed an adequate diet and were kept on an alternate light and dark schedule of 12 hours with the light period extending from 8 A.M. to 8 P.M.

Thin blood smears from the monkeys were made at one to three hourly intervals during many segmentations and at 4 to 10 hourly intervals between segmentations during the patent infection. At each interval several smears were made. Half of them were dried in air and the other half were momentarily dipped in ether, as suggested by Boyd (1939), to facilitate counting merozoites.

To study the length and synchronism of the asexual cycle, a random sample of 50 to 100 asexual parasites from closely-spaced blood films was differentially classified into the following 5 groups: small rings, intermediate and large uninucleated trophozoites, and 2 to 4 and 5 or more nucleated schizonts. The first three categories were separated by size. Intermediate stages were roughly from one-sixth to one-quarter the area of a red cell. Small rings were smaller and large uninucleated stages were larger than intermediate trophozoites. Macro- and microgametocytes were also classified. To represent the asexual cycle, the percent of multinucleated stages (classes 4 and 5 together) are used in the graphs (see dotted line) instead of small rings, as used in the work on *P. cynomolgi*, because multinucleated stages of *P. knowlesi* were not differentially filtered out in the tissues to the extent that those of *P. cynomolgi* were.

Merozoite means were ascertained from counts of the merozoites in daily samples of 25 or more segmenters throughout the course of various infections. Usually half the segmenters were located on ether films and half on regular blood films at two intervals (usually two hours apart) during maximal segmentation. Standard errors were calculated for each daily mean throughout the various infections. They were usually <0.2 but sometimes were 0.3 or 0.4. Segmenters were located as in our previous publication (1947), and merozoites were only counted in segmenters in which the daughter merozoites were completely separated.

Parasite counts, length of the asexual cycle and merozoite means per segmenter were further used to study reproduction-inhibiting and parasiticidal effects of immunity (cf. infections with *P. brasilianum*, W. H. and L. G. Taliaferro, 1944). For example, when an average of 10 merozoites is produced per segmenter for a given asexual cycle, and the parasitemia increases during segmentation from 1 to 8 per 10 red cells and for the entire cycle to the beginning of the next segmentation from 1 to 3 parasites, the following conclusions are justified: Of the 10 merozoites produced (merozoite mean) per segmenter, 8 survived and penetrated new red cells (conversely, the extracorpuscular or merozoite death rate was 2) whereas of the surviving merozoites only 3 developed fully (conversely the intracorpuscular death rate of schizonts was 5). Furthermore, the over-all parasite survival and death rates were 3 and 7 parasites, respectively. In knowlesi infections, large stages are frequently retained in the tissues. For this reason, we computed over-all survival and death rates at each asexual cycle from the peaks in parasite counts of the ring stages of consecutive asexual cycles just after segmentation. For the same reason, it was frequently impossible to differentiate the intra- and extracorpuscular death rates because the parasite count before segmentation was abnormally low. We only computed these values when the percentage of multinucleated forms appearing in the blood was comparatively high and believe they give approximate trends. The

percentages of gametocytes were invariably so low they were disregarded in the computations.

In considering the over-all survival rates of *P. knowlesi*, it should be kept in mind that a net survival of one parasite per segmenter results in a static parasitemia if the parasitemia is measured daily at the same stage of the asexual cycle, i.e., if intracorpuscular death is ignored. Occasionally, less than one parasite per segmenter survives with consequent decreases in parasitemia. These decreases are always clearly more than the usual intracorpuscular death of developing parasites and are sometimes designated parasite declines. The length of the asexual cycle has to be considered in computing the basic rate of reproduction, but, since it was essentially 24 hours long, we have limited most of our computations to merozoite production and death and survival of parasites per segmentation.

EXPERIMENTAL RESULTS

The ordinarily fatal infection of *P. knowlesi* in rhesus monkeys is characterized by a 24-hour asexual cycle as has frequently been reported and as is shown in figure 1.* Under the 12-hour light and dark schedule maintained in our animal quarters, mature segmenters usually could be found during the late morning in the early part of the patent infection, reached a peak in numbers around noon and had practically disappeared 4 hours later. Thereafter, around 5 P.M., practically all the forms were small ring stages. As the infection progressed, the cycle on the average was delayed and somewhat prolonged as is explained under the individual infections. Red cells did not become stippled with Giemsa stain or appreciably enlarged, but were distorted as reported by Mulligan (1935).

The course of infection of *P. knowlesi* was studied in 18 monkeys. Five of the infections will be described in detail, as representative of the variations found, and the other 13 will be more cursorily dealt with.

Rhesus 1.—The data from this infection with respect to the rate of reproduction and survival and death rates of parasites are graphed in figure 2. They include the percent of multinucleated stages, merozoite mean per segmenter, parasite count, and net survival (shaded area) and net death rates (clear area under the merozoite mean) of parasites for the last 5 days of the infection. To simplify the following descriptions, the phrase, "after two, three, etc., days" will be synonymous with "on the second, third, etc., day." Both will refer to days after infection considering the day of infection as the zero day, unless specifically stated that a given interval designates some stage of the patent infection.

A few parasites appeared in the blood of rhesus 1 two days after an intravenous injection of parasitized blood, and the monkey died on the 7th day with an overwhelming parasitemia of 11 parasites per 10 red cells.

During the asexual cycle of 24 hours on the third day of infection, maximal segmentation occurred around noon but multinucleated forms only reached a peak of 20% in the blood, a finding which indicated that large forms were retained in the inner organs. During successive segmentations, multinucleated forms reached progressively higher peaks, and the asexual cycle was progressively delayed and somewhat prolonged. Thus, on the last day of the infection, segmentation extended from noon to 10 P.M. with the peak around 6 P.M. At 5 P.M., 73% of the population were multinucleated. The differential retention of large forms in the tissues was intermediate in amount between that occurring in infections with *P. cynomolgi* and *P. brasilianum* (W. H. and L. G. Taliaferro, 1944 and 1947).

The merozoite mean per segmenter

* This figure shows the time relations observed during the first part of most infections. It was, however, prepared from the late part of an infection, when maximal segmentation was delayed approximately three hours. The delayed time is shown in the reproduction given in Aberle (1945).

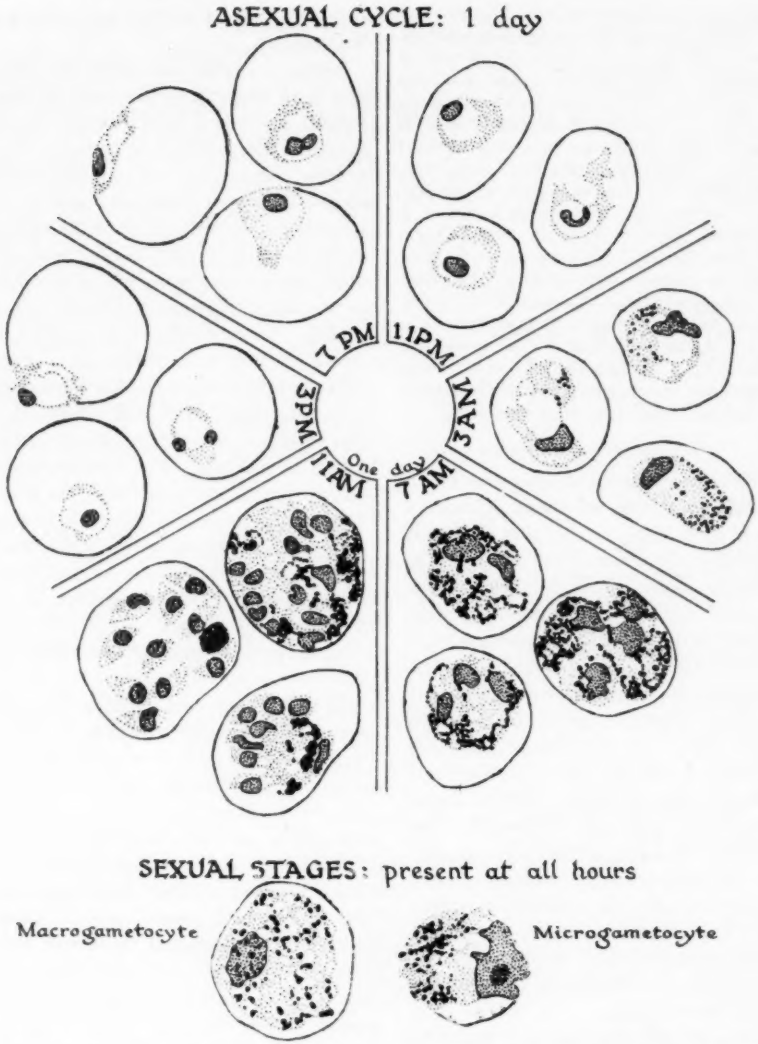

FIG. 1.—Representation of the 24-hour cycle of *P. knowlesi* constructed from outlines made with the camera lucida. In the parasites, the cytoplasm is lightly stippled, the nucleus is darkly stippled and the pigment is indicated by black granules or masses. ×3000.

See footnote on page 109.

was high for three days ($9.9-11.0\pm0.4$) and then decreased until it was 8.5 ± 0.2 at the time the monkey died. During the latter part of the infection, morphologically abnormal forms were present. They were similar to crisis forms as described by us in other malarias (1934a, 1944 and 1947) and the aberrant schi-

zonts described in this infection by Afridi (1938). The number of merozoites produced by the segmenters in the various samples ranged from 6 to 16 with from 40 to 92% of the segmenters containing from 7 to 10 merozoites. The decrease in mean on the last two days of the infection was reflected by a decrease in segmenters producing a large number (11 to 16) of merozoites. During the first of the infection such segmenters formed 35 to 60% of the segmenters whereas on the last two days of the infection they formed 12 and 4%, respectively.

During each 24 hours, the parasite count increased at first and subsequently decreased. This step-like progression of the parasitemia, as shown in figure 2, is typical of every synchroncusly reproducing plasmodium. It is the resultant of the periodic division of the parasite as modified by death and phagocytosis of varying proportions of the resulting progeny (see Taliaferro and Mulligan, 1937, for a literature review and experimental data).

Daily parasite counts of ring stages for the last 6 days of the infection were 0.006, 0.046, 0.29, 1.76, 5.1 and 11, respectively. These counts gave rates of increase of 7.7, 6.3, 6.0, 2.9 and 2.1. Since the merozoite means beginning on the third day of infection were 10.6, 9.9, 11.2, 9.1 and 8.5, the percent survival of parasites for the last 5 days was 72, 64, 54, 32 and 25%, respectively. Thus, the percent survival of parasites steadily decreased and, conversely, the percent death of parasites steadily increased from 28 to 75%.

Rhesus 2.—The infection in this monkey is graphed in figure 2. It was essentially similar to the infection in rhesus 1 with respect to the parasitemia, asexual cycle and retention of large forms in the blood. The infection differed from the one in rhesus 1 only in the following respects. (1) Parasites appeared in the blood one day later and the parasitemia was slightly less intense. (2) The merozoite mean stayed consistently higher ($11.2–11.9 \pm 0.4$) during the first three days and decreased more precipitously on the 4th day (7.8 ± 0.2 as compared to 9.1 ± 0.3). The high merozoite means were reflected by 52 to 68% of the segmenters having 11 or more segmenters, and the low ones, especially on the 6th and 7th days, by 72 and 88% of segmenters having only 7 or 8 merozoites. (3) One hundred per cent of the merozoites infected red cells and grew to maturity on the first and second day of the patent infection. The progressive decrease in survival rate of parasites beginning either with 100% as in this monkey or with <100% as in rhesus 1 was characteristic of the acute rise and crisis of infections of *P. cynomolgi* but not of *P. brasilianum* (W. H. and L. G. Taliaferro, 1944 and 1947) or of *P. lophurae* (Reilly et al., 1949).

Rhesus 3.—This infection, which is graphed in figure 2, exhibited the following two interesting differences.

1. Higher percentages of multinucleated forms occurred just before each segmentation. They varied from 54% around 1 P.M. after 4 days (second day of the patent infection) up to 80% at 8 P.M. on the last day of the infection. Parenthetically, it may be noted that the difference in time in the peak of multinucleated forms shows the delay in the asexual cycle of the majority of the population during the course of the infection as has already been described for the other two monkeys.

The large percentage of multinucleated forms at each segmentation allowed us to approximate the survival and death of merozoites and intracorpuscular schizonts during each asexual cycle. For example, the synchronously segmenting parasite population during the 5th asexual cycle gave the following

parasite counts for the 24-hour period: 0.6 per 10 red cells (ring stages) at 5 P.M. 0.45 (multinucleated stages) at 9 A.M. the next morning and 3.6 (ring stages) at 8 P.M. that evening. These counts gave a rate of increase of 8 during segmentation and an over-all rate of increase of 6. In addition, the merozoite mean of 100 segmenters between 5 and 8 P.M. was 8.9 ± 0.15. Therefore, of 8.9 parasites produced, approximately 8 (90%) survived as merozoites to infect new red cells and approximately 6 (67%) of the intracorpuscular stages grew to maturity. Stated in terms of death of parasites, 10% died as free merozoites and 23% died during intracorpuscular growth. The death of free merozoites was probably somewhat higher and that of intracorpuscular parasites lower because some multinucleated stages were undoubtedly localized in the tissues. This is borne out by the fact that, from blood samples made hourly during the day, multinucleated stages reached a peak of 81% at 3 P.M., whereas 100% multinucleated stages occurred in infections of *P. brasilianum* for an appreciable length of time when there was only one brood of parasites. Data for the other asexual cycles during the infection indicated (1) that practically all of the

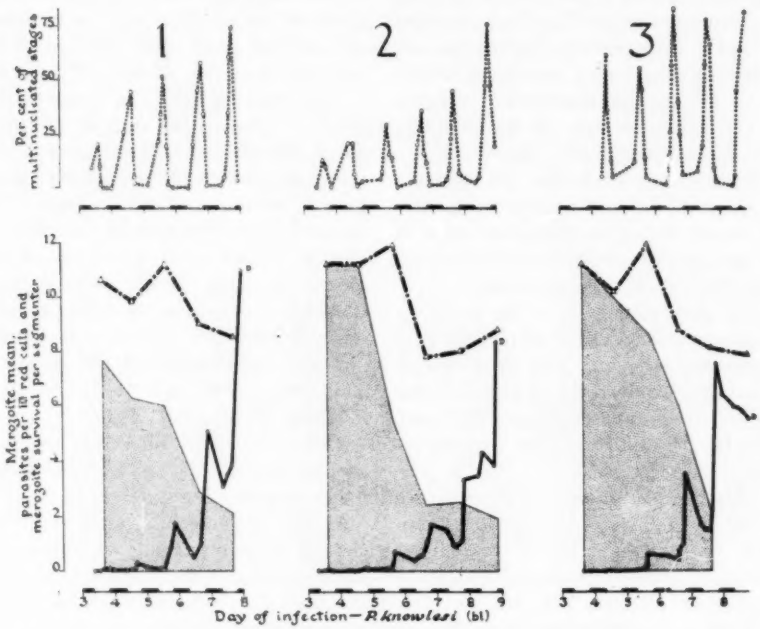

FIG. 2.—Blood-induced progressively acute *P. knowlesi* infections in rhesus monkeys 1, 2 and 3 as shown by the rate of reproduction, i.e., length of the asexual cycle and merozoite mean per segmenter, parasitemia and survival and death of parasites.

The midpoint of segmentation occurs just after the peak in multinucleated stages (dotted line). Merozoite mean, dot and dash line; parasitemia, solid line; over-all parasite survival, i.e., merozoite survival per segmenter, shaded area; over-all parasite death per segmenter, clear area under merozoite mean; D, dead; daily periods of light and dark, light and heavy segments of the base line.

merozoites infected red cells during the two preceding asexual cycles, and 50 and 14%, respectively, infected red cells at the last two, and (2) that of the merozoites that infected red cells practically all grew to maturity during the two preceding ones, and 25 and 11%, respectively, grew to maturity during the last two.

2. As a result of the slightly higher survival rates in this monkey, the parasitemia increased more rapidly and reached a peak of 7.5 on the 7th instead of the 8th day as in rhesus 2. At this point, the monkey did not die. It died on the following day as the parasitemia was approximately 5.5 per 10 red cells.

Rhesus 4.—As shown in figure 3, this monkey lived longer (10 instead of 7 to 8 days) than the preceding monkeys in spite of the fact that its blood contained a few parasites one day after infection and the terminal infection was about the same. The difference was possibly associated with a retardation on the 5th and 6th days of the acute rise of the infection during which the over-all survival rate of parasites was only 11%. This rate then rose to 100% just before the monkey died. Peaks in multinucleated stages just before each segmentation were low at first and higher later. The merozoite mean per segmenter decreased to a lower level in this monkey than in any of the preceding ones.

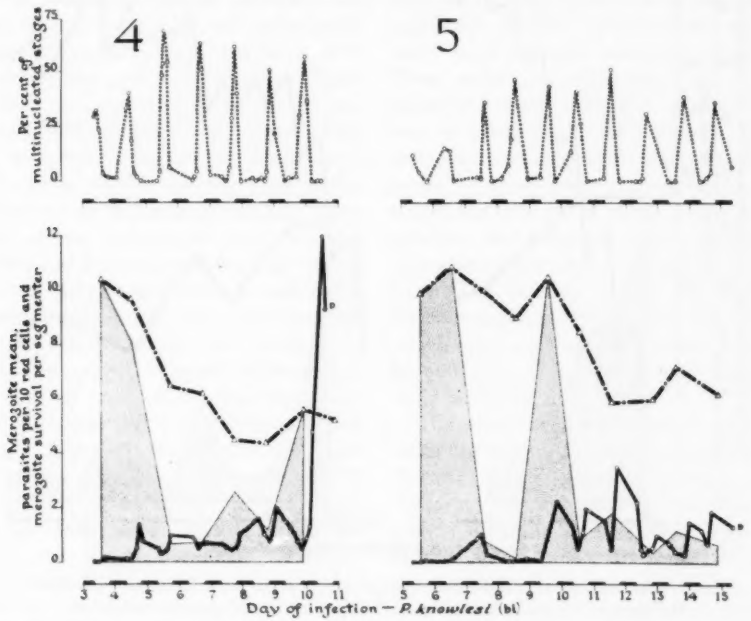

FIG. 3.—Blood-induced intermittently acute *P. knowlesi* infections in rhesus monkeys 4 and 5. For explanation, see legend in figure 2.

Survival of extracorpuscular merozoites was calculated throughout. In general, survival rates of both extra- and intracorpuscular asexual stages decreased on the 6th day to the same low level (<15%) attained on the last day in rhesus 3 and rose sharply (55 and

58%) on the next day. On the 8th day, only the intracorpuscular survival rate decreased (27 and 60%). At the final segmentation, they both increased to 100%. We may conclude, therefore, that the immune response of the host was more actively mobilized but was not sufficiently effective to avert a fatal outcome.

Rhesus 5.—This infection was similar to the one in rhesus 4 in that it was not progressively acute. As compared to rhesus 4, the parasitemia was more drastically suppressed and never rose higher than 3.5 per 10 red cells, the merozoite mean remained higher during the first of the infection, and the overall parasite survival fluctuated to a greater extent (fig. 3).

Other rhesus monkeys.—Infections initiated with intravenous injections of infected blood were studied in 13 other monkeys. Eight of these resembled the type found in monkeys 1, 2 and 3 and we have designated them progressively acute fatal infections. Four were similar to the type in rhesus 4, and the remaining one approximated that in rhesus 5. These we have designated intermittently acute fatal infections because the parasitemia failed to increase progressively. The 11 (61%) progressively acute and the 7 (39%) intermittently acute infections are depicted in two composite graphs in figure 4. The parasitemia curves in this figure are graphed on a logarithmic scale instead of an arithmetic one and the prepatent peri-

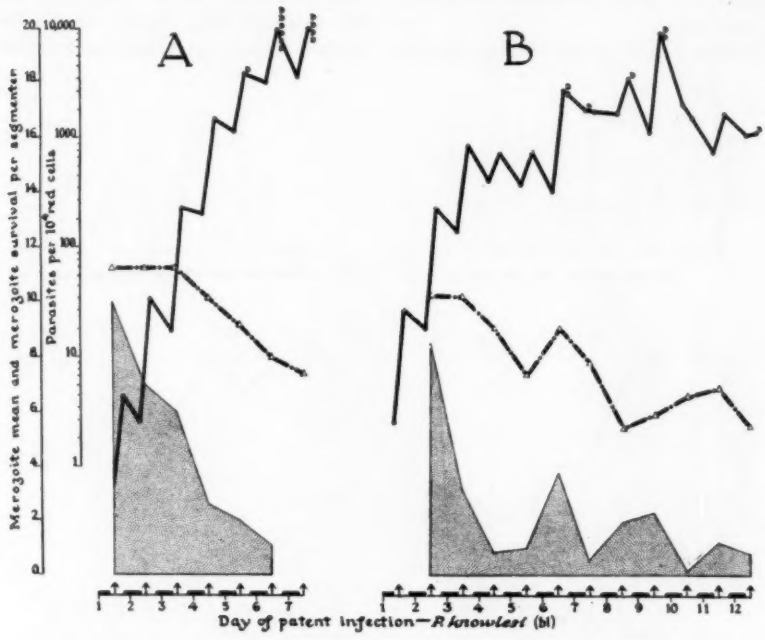
A
B
Merozoite mean and merozoite survival per segmenter
Parasites per 10⁴ red cells
Day of patent infection—P. knowlesi (bl)

FIG. 4.—A: Mean of 11 blood-induced progressively acute fatal *P. knowlesi* infections. B: Mean of 7 blood-induced intermittently acute fatal *P. knowlesi* infections. Arrows indicate asexual segmentation. For other explanations, see legend in figure 2.

ods are omitted. Intervals in the two following sections, therefore, will refer to the patent infection and not, as formerly, to intervals after infection unless expressly stated otherwise.

Progressively acute fatal infections.—Many of the characteristics of this type of infection, as described in the following paragraphs, are shown in the composite graph in figure 4A.

The prepatent period, following an intravenous injection of parasitized blood, extended from 0 to 4 days. The length of this period is a function of the number of parasites injected (as reported by Coggeshall and Eaton, 1938; cf., however, Brug, 1934). The merozoite mean was invariably high at the beginning of the patent period. Since most of the infections were initiated with blood from a donor monkey during the last part of infections in which the merozoite mean was low, it follows that the merozoite mean rose during the prepatent period. It is not unlikely that survival of extra- and intracorpuscular parasites was 100% during most if not all of this period. Moreover, the asexual cycle was usually synchronous and 24 hours long at the beginning of the patent infection. The straggling segmenters from the donor monkeys, therefore, must have died, accelerated their rate of division or skipped a segmentation and joined the succeeeding brood.

The patent period lasted 5 days in one monkey and 6 or 7 days in the remainder. The parasitemia, aside from characteristic decreases during intracorpuscular growth, progressively increased throughout all infections except two (cf. rhesus 3, fig. 2). In the latter, the terminal decrease may have represented the beginning of a parasite decline. Peaks of 7 to 12 parasites per 10 red cells were reached in all.

The asexual cycle was essentially 24 hours at the beginning of the patent infection with one clearly defined brood of parasites. Rings were liberated by segmentation approximately from 11 A.M. until 5 P.M. (fig. 5). This interval is about two hours shorter than that found by us (1934a) in the quartan parasite *P. brasilianum*. As the infection progressed, maximal segmentation occurred later, and rings were liberated over a longer period (cf. the 2nd with 6th day in fig. 5) due to straggling, probably injured, segmenters which took more than 24 hours to develop. Thus, a few parasites may have progressively taken a few more hours each day to grow until, for example, they may have segmented 7 while the majority of parasites were segmenting 8 times. On the other hand, there were always a few parasites which continued to segment in 24 hours. In addition, we found one parasite population with a delayed segmentation in two infections which regained its normal segmentation by speeding up its growth and division to 21 hours on the average.

The most atypical sequence in the asexual cycle which we encountered is shown in the second column of figure 5 by data on three consecutive cycles of the patent infection in monkey 6. This infection had a prepatent period of two days and the monkey died at 6 A.M., i.e., two hours after the last determination shown on the graph. On the second day of the patent infection, retention of large and multinucleated schizonts in the tissues was marked (peak of 18% in the blood); the midpoint of segmentation, i.e., 50% rings, occurred around 8 A.M.; segmentation extended from about 4 A.M. to noon as determined by the simultaneous occurrence of multinucleated stages and rings in the blood; and the asexual cycle was approximately 24 hours long since segmentation occurred somewhat similarly on the first day of the patent infection. On the

third day, multinucleated stages occurred in large numbers (peak of 56%) in the blood; the midpoint of segmentation occurred around 1 P.M.; segmentation extended from about 5 A.M. to 5 P.M.; and the asexual cycle was about 29 hours. On the 4th day, multinucleated stages occurred in large numbers (peak of 59%) for a long time; the midpoint of segmentation occurred around 7 P.M.; segmentation took place from about 8 A.M. to midnight; and the asexual cycle was about 30 hours long.

Unquestionably, cycle delays and lengthenings are associated with injury to many of the parasites (crisis forms, etc.) On the other hand, however, no matter how deranged or delayed a cycle becomes, a few schizonts segment in the early afternoon. A cycle could regain its original periodicity and normal time of segmentation by a selection of uninjured parasites or a recovery of injured ones in, for example, a new host.

During the first and second asexual cycle in some patent infections, large uninucleated and multinucleated schizonts were scarce (rhesus 6 in fig. 5). In other infections at this time and during all infections thereafter, the percentage frequency of multinucleated schizonts was over 50% but was rarely higher than 85%. Since populations consisting of 100% multinucleated stages were present for several hours of the three-day cycle when one brood of *P. brasilianum* was segmenting, we expected to find a similar high percentage at least for an hour in some asexual cycles in *P. knowlesi* (this strain never had more than one brood). Failure to find such a sequence, though hourly slides were examined during several segmentations in several infections, led us to conclude that large uninucleated and multinucleated stages, including segmenters, were differentially retained in some of the inner organs. This conclusion was borne out by differential determinations of spleen, liver and bone marrow counts. Large stages formed higher proportions (up to 4-fold) of the parasite population in the tissues (especially in the spleen and liver) than in the blood in 6 of 7 monkeys which died with segmenters in their blood. Even higher proportions would probably have been found had monkeys been killed during the early acute rise. Parenthetically, a distinction should be made between a higher percentage of multinucleated stages in the tissues than in the blood and a higher occurrence of parasites of all stages in the tissues than in the blood. This latter condition is found at times in *P. knowlesi*, *P. brasilianum* and *P. gallinaceum*, and is higher in infections of *P. brasilianum* than of the other two (W. H. and L. G. Taliaferro, 1934a; W. H. Taliaferro, 1948b). In infections of *P. knowlesi*, there were usually about 10 to 40% more parasites of all stages in the tissues than in the blood at the death of the monkeys (cf. Taliaferro and Mulligan, 1937, for earlier parts of the infection).

Changes in the merozoite mean and in the range of merozoites during the course of this type of infection may be seen in table 1A, which was compiled after examining over 2400 segmenters. Segmenters usually formed from 6 to 16 merozoites during the first part of the infection, but one segmenter with 18 merozoites was found on the 4th day in one monkey. Segmenters with an odd number of merozoites undoubtedly occurred. They generally formed from 8 to 12% of the various samples and became more numerous as the infections advanced. Segmenters formed from 4 to 12 merozoites on the last two days.

Extremes in inhibition of reproduction were found in monkeys 1 and 8. In the first animal the merozoite mean was reduced from 10.7 to 8.5 and the asexual

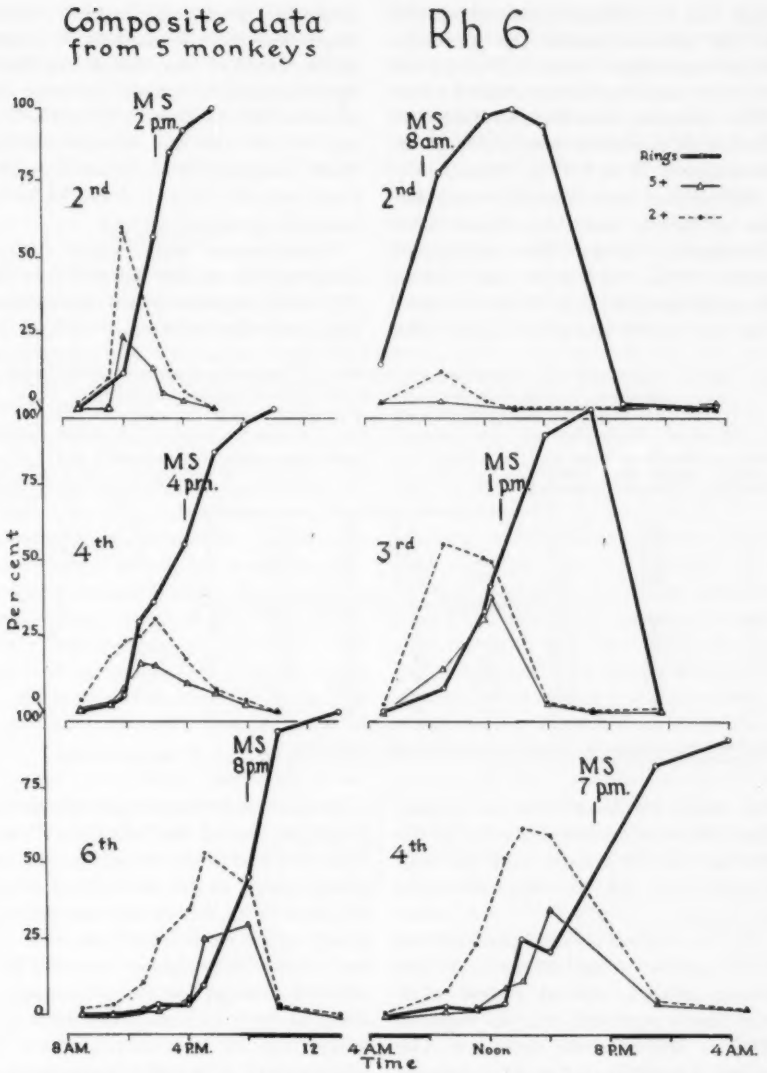

FIG. 5.—Left hand column. Three segmentations on the 2nd, 4th and 6th days of the patent infection of progressively acute infections as shown by average percent of rings, 5 nucleated and multinucleated stages from 5 monkeys.

Right hand column. Three consecutive asexual cycles on the 2nd, 3rd and 4th days of the patent infection of rhesus 6 showing the maximal extent to which segmentation may be delayed and prolonged.

MS=approximate midpoint of segmentation.

cycle was lengthened about two hours. In the second animal the merozoite mean was reduced from 11.2 to 6.4 and the cycle was lengthened about 4 hours. These animals, therefore, showed a reduction in merozoite production of approximately 25 and 50%, respectively.

Merozoites were counted in segmenters on regular and ether-dipped blood films made at the same time and on both kinds of films 2 to 5 hours apart during the same asexual cycle. Means obtained from the regular and ether-dipped films often varied by about 10%, but no constant difference occurred. Similarly, the difference between early and late segmenters from 14 segmentations was 0.45 ± 0.24.

The over-all survival of parasites, as determined by rates of increase for each 24-hour asexual cycle at a time when small forms occurred, was as much as 100% at first in some monkeys. The average was 89% in the 11 monkeys. From this high point, it decreased at every asexual cycle until it was 11 to 30% (average = 13%). Reciprocally, the over-all death rate progressively increased. From the death rates at and between segmentations, the tentative conclusion may be reached that the progressively increasing over-all parasite death was more dependent on changes in the death of free merozoites than of intracorpuscular stages. Average merozoite death rates were 0, 0, 40, 66, 76 and 73 for the last 6 segmentations, while average intracorpuscular death rates were 11, 38, 7, 9, 3 and 14 for corresponding segmentations.

Gametocytes were scarce. Macrogametocytes usually formed less than 1% of the population and microgametocytes were far fewer.

TABLE 1.—*Composite merozoite means per segmenter and percentage frequency distributions of segmenters with the following number of merozoites throughout progressively acute and intermittently acute fatal infections with* P. knowlesi.

Day of patent infection	Number of segmenters examined	Merozoite mean per segmenter	Number of merozoites per segmenter*							
			4	6	8	10	12	14	16	18
A. Progressively acute infections in 11 rhesus monkeys										
1–3	418	11.2		1.4	17	26.6	*33*	18	4	
4	557	10.1		2.8	*36*	27	18	12	4	0.2
5	592	9.3	0.2	3.0	*46*	30.5	16	4	0.3	
6	576	8.0	2	21.5	*52*	20	4	0.5		
7	300	7.4	5.5	28.0	*55*	10	1.5			
B. Intermittently acute infections in 7 rhesus monkeys										
2– 3	190	10.2		3	28	*33*	24	9	3	
4	184	9.2		4	41	*42*	10	3		
5	276	7.4	3	15	*50*	24	5	3		
6	328	9.0	3	15	*37*	25	12	8		
7	236	7.8	11	20	*43*	15	8	3		
8– 9	200	5.8	22.5	*57.5*	19.5	0.5				
10–11	127	6.8	10	42.5	*44*	3	0.5			
12	50	5.5	40	*40*	18	2				

Figures in italics indicate the highest percentage frequency on each day.
* Segmenters with odd numbers of merozoites from 3 to 15 are included in the next larger even number class.

Intermittently acute fatal infections.—A comparison of the infections in monkeys 1, 2 and 3 with 4 and 5, the composite graph in 4A with 4B and table 1A with 1B indicates that the intermittently acute fatal infections were similar to the progressively acute type of infection except for the following: (1) Some of the infections lasted a few days longer. (2) The parasitemias were intermittently acute with peaks in numbers varying from 3.5 to 12 per 10 red cells. (3) The merozoite means per segmenter were about 10% lower at the beginning of the patent infections with fewer 16-nucleated segmenters. Thereafter, they fluctuated in a general downward direction. Toward the end of

the infections, there was ordinarily a range of from 3 to 10 merozoites per segmenter. (4) The recurring low merozoite means were generally associated with crisis forms, suppression of the parasitemia, high death rates and a tendency for the asexual cycles to be slightly longer.

DISCUSSION

Much of the present work deals with the survival of merozoites formed at each segmentation as determined by a comparison of the merozoite mean with the factor of parasitemia increase. It should be noted, therefore, that our results differ in certain essential respects from those on the same infection by Brug (1934). He concluded that, although many parasites disappear during the infection as a whole, the blood is periodically flooded with parasites during the patent infection. Thus, in one blood-induced infection lasting 13 days, a "tremendous loss of parasites" was indicated by calculations from the number of parasites injected, assuming that no parasites died and that the original number increased each day by a factor of 9 (the approximate merozoite mean per segmenter), whereas the parasitemia actually increased 31.8 times daily on the average for the three asexual cycles of the patent infection. Such results would invalidate our calculations on the survival and death of parasites. Afridi (1938) also noted large sudden increases in parasitemia at the beginning of the patent infection, but he found the same number of parasites in the spleen and the blood on the day before such sudden increases. Blood and spleen films at 2 to 4 hour intervals throughout a cycle in two monkeys indicated that the multinucleated forms were partially retained in the spleen but not the rings. He also obtained evidence that the forms appearing in the blood lagged 2 to 4 hours behind those in the spleen.

Our work indicates that the retention of forms in the internal organs is limited to the larger parasites and is greatest at the beginning of the infection. Furthermore, we did not usually obtain large rates of daily increase when we used rings to obtain the factor for over-all survival. In fact, we only found a few cases in which the factor was greater than the merozoite mean. Thus, in monkeys 1 through 5, inclusive, in which the data were most extensive, only 6 determinations showed a higher factor of increase than the merozoite mean and amounted, respectively, to 0.1, 0.3, 1.2, 1.7, 2.1 and 2.1. Judging from the standard errors of the merozoite means, the smaller values were not statistically significant and the larger ones were invariably obtained when parasite counts were very low, i.e., at the beginning of the patent infection. We have, therefore, considered such discrepancies insignificant and have indicated 100% survival in figures 2 and 3 for these readings. We obtained, however, a few higher factors of increase in determinations involving parasite counts before and after segmentation than could be accounted for by the merozoite mean. The most marked one was a 36-fold increase in the parasite count on the 4th day after infection in rhesus 1, when the merozoite mean was 9.9. Here, as in the previous instances, the parasite counts were not reliable because the infection was just appearing in the blood. In addition, the parasite count before segmentation was probably too low because multinucleated stages only reached a peak of 44%. The addition of comparatively few large segmenters in absolute numbers to the blood would raise the parasite count before segmentation sufficiently to give a rate increase in rings compatible with the merozoite mean. Since, therefore, the majority of our

calculations on rates of increase were within the expected range of the merozoite mean and the others could be accounted for by technical difficulties or the differential retention of large forms in the tissues, we doubt Brug's contention that the peripheral blood is flooded with parasites and are at a loss to explain his high rates of increase. As we previously have pointed out, however, when large forms are differentially retained in the tissues, number counts before segmentation cannot be relied upon to compute the average number of merozoites that survive the passage of merozoites from one erythrocyte to another.

Results reported in this paper indicate that schizonts of *P. knowlesi* produce on an average 10 to 11 merozoites in the 24-hour asexual cycle at the beginning of the patent infection. During the next asexual cycle or after several cycles, they produce fewer merozoites until a low value of 6 (occasionally 4) is reached. Thus, the rate of reproduction, as measured by the merozoite mean, decreases more or less progressively during each infection until it is only 50 to 75% of the original rate.

Of the merozoites produced, all or nearly all survive and complete their development early in the infection, but this over-all survival progressively decreases as the infection progresses until only one to two merozoites per segmenter or about 10% of the parasites complete their development. Thus, the production and over-all survival of parasites progressively decrease, but the latter decreases at a faster rate. In intermittently acute infections, the production and over-all survival of parasites may reach the same low values one or two days earlier, but thereafter may fluctuate to varying degrees.

The differential retention of multinucleated parasites in the tissues during the early patent infection is more pronounced in infections of *P. cynomolgi* than of *P. knowlesi*, and is negligible in infections of *P. brasilianum* and *P. gallinaceum* (W. H. and L. G. Taliaferro, 1934a, 1944, 1947 and 1949). Cannon (1941) suggested that retention of large forms of *P. falciparum* in man may be due to their localized agglutination in the smaller capillaries by immune agglutinins. This suggestion does not seem applicable to *P. knowlesi* and *P. cynomolgi* because immune agglutinins would tend to be stronger late rather than early in an infection.

The asexual cycle is essentially 24 hours long as previously determined by Sinton and Mulligan (1933), Mulligan and Sinton (1933), Brug (1934), Mulligan (1935) and others. Factors involved in the synchronous development of plasmodia depend not only upon the parasite but upon conditions in the host such as activity, feeding and immune phenomena (cf. especially Boyd, 1929a and b and 1933; Stauber, 1939; and W. H. and L. G. Taliaferro, 1934b, 1944, 1947 and 1949).

Prolongation of segmentation and lengthening of the cycle by one to several hours (cf. Afridi, 1938) do not lead to the formation of extra broods possibly because the cycle is 24 hours long. Where extra broods are formed, i.e., in *P. brasilianum*, *P. cynomolgi* and *P. gallinaceum*, the cycle is more than 24 hours (W. H. and L. G. Taliaferro, 1934a, 1944, 1947 and 1949).

The acceleration of the asexual cycle as noted occasionally during the course of this work seemed to be a wholesale realignment of segmentation to restore the original timing. It may have occurred more often than noted here since its establishment rests upon the examination of hourly slides during periods of segmentation. We only undertook such a study for a few segmentations in some

monkeys. Jolly et al (1937) postulated the occurrence of 20-hour cycles to explain a return of the asexual cycle to its original timing after a delay. They did not, however, make smears at closely-spaced intervals. Such an acceleration was noted previously by the junior author (1928) after refrigeration of *P. cathemerium*. It is interesting that this species also has a 24-hour cycle.

Degenerate parasites occur during the last half of knowlesi infections as noted previously by Alfridi (1938). They are probably associated with parasiticidal effects of acquired immunity since they have been described in several other species of plasmodia, chiefly at the time of the intense immune reaction associated with the crisis (W. H. and L. G. Taliaferro, 1934a, 1944 and 1947; Thompson, 1944).

Elsewhere the senior author has presented a series of schematic diagrams (fig. 6) of several malarias showing marked differences in merozoite production and over-all survival and death rates. In interpreting these diagrams, it should be kept in mind that an over-all survival of one parasite per segmenter during any asexual cycle results in an essentially static population, a greater survival in an increasing parasitemia and a lesser survival in a decreasing parasitemia.

In many blood-induced infections with *P. brasilianum* in monkeys, the

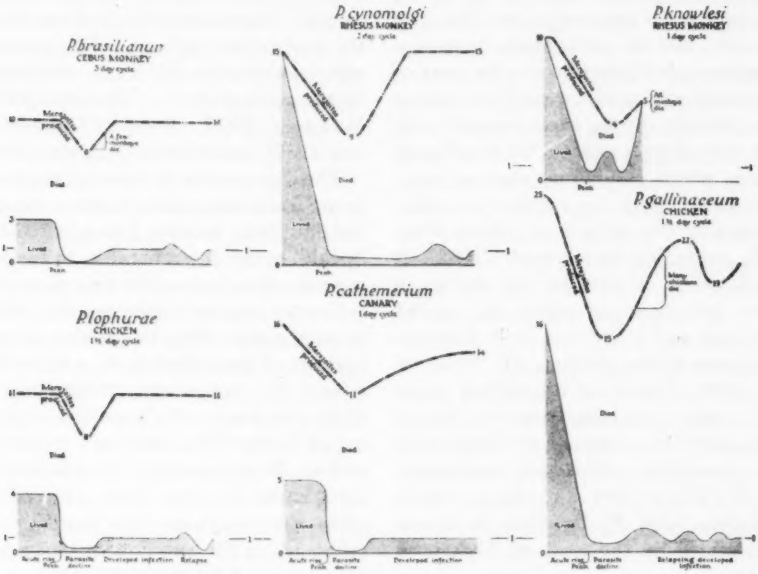

FIG. 6.—Schematic diagram of merozoite production and survival and death per segmenter in several species of simian and avian plasmodia.

For ease of comparison the stages of the infections, i.e., acute rise, parasite decline and developed infection are arbitrarily indicated by intervals of the same length in all infections although they may vary in length over a wide range. In the diagram for *P. knowlesi*, which was made from rhesus 4 (fig. 3), "peak" indicates the point at which the mounting parasitemia is first checked. (From Taliaferro, 1948a).

merozoite mean per segmenter remains more or less constant throughout the infection. During the acute rise of the infection, approximately 7 out of 10 merozoites produced die. The over-all survival of about three parasites at each asexual cycle gives a typical exponential population increase. During the crisis, the survival is reduced to less than one parasite, and the parasitemia decreases. The developed infection may be more or less static with a net over-all survival of one parasite during each asexual cycle but more often is punctuated by relapses during which varying numbers of parasites in excess of one survive. *P. cathemerium* in the duck may behave similarly according to Simpson (1948). A modification of this picture occurs in some infections in cebus and spider monkeys and is the rule in *P. lophurae* infections in the chicken (cf. Reilly et al., 1949). Following an intense acute rise, a sharp parasite crisis may be accompanied by a temporary decrease in the merozoite mean per segmenter (fig. 6). This picture may also be seen in infections with *P. floridense* in lizards as revealed by the work of Thompson (1944).

In infections produced by *P. cynomolgi* in rhesus monkeys, the merozoite mean per segmenter and the survival of parasites is high during the first asexual cycle of the patent period, but the former decreases to approximately two-thirds of its original value by the end of the crisis and the latter decreases even more rapidly. Thereafter, the merozoite mean returns to its original value and maintains this rate during the developed infection, whereas the survival rate remains low. These conditions result in a parasitemia during the acute rise which increases at a diminishing rate.

P. cathemerium resembles *P. cynomolgi* in the number of progeny produced, although the original high rate is not usually regained during the developed infection, and resembles *P. brasilianum* and *P. lophurae* in survival rates. From the fact that the production of merozoites constantly decreases during the acute rise while the absolute number that survives is constant, a smaller proportion of parasites die as the acute rise progresses. This remarkable combination has been established by the work of L. G. Taliaferro (1925), Hartman (1927), Boyd (1929a and b and 1939) and Boyd and Allen (1934).

The diagram for *P. knowlesi* shown in figure 6 is a schematic representation of the data from monkey 4 in figure 3. The "peak" in the diagram refers to the first parasite decline, i.e., the high point just before an expected increase first failed to materialize. This is not the peak in number of the infection as a whole but orients the immunological phenomena with infections which recover by an initial crisis. This infection at first as well as all progressively acute infections are similar to the acute rise of *P. cynomolgi* infections. The fundamental difference in the subsequent characteristic course of infection may be that, in cynomolgi infections, immunity reduces and maintains the over-all survival to less than one parasite per segmenter long enough to control the infection, whereas, in knowlesi infections, it fails to reduce over-all survival to this level or fails to maintain it.

The acute rise of *P. gallinaceum* in the chicken is similar to the first part of those *P. knowlesi* infections in which there is less than a 100% survival and differs thereafter in that merozoite production markedly increases, while parasite survival increases little if at all. In making this comparison it should be noted, however, that the undulations in the merozoite mean in gallinaceum infections, even if blood-induced, es-

pecially during the developed infection, may be due to recurring showers of exoerythrocytic forms which possess a different mean from the blood stages.

The foregoing infections are similar in the following two relative respects: (1) Large numbers of intra- and extracorpuscular parasites die during innate immunity (the early acute rise of each infection) and even larger numbers die as acquired immunity is superimposed on innate immunity (the late acute rise, parasitological crisis and immediately ensuing developed infection). It is noteworthy that all of the parasites only survive for several asexual cycles during the early part of a few of the foregoing infections. Black (1948), however, reported that the parasitemia for several cycles in two infections with the New Guinea strain of *P. falciparum* showed a rate increase of 22. This may indicate close to 100% survival, but no merozoite means were given for the specific segmentations studied. (2) The length of the asexual cycle tends to remain constant for a given species except during the latter part of knowlesi infections and during intense crises in several other malarias. In the latter case one brood of parasites may realign into multiple broods of varying proportions.

On the other hand, the infections fall into two categories with respect to relative changes in the merozoite mean and death rate per segmenter. These are exemplified by *P. lophurae* and by *P. cynomolgi*. In infections of *P. lophurae* in chickens, the merozoite mean remains more or less constant during the entire infection except for a temporary decrease at the time of the parasitological crisis. This sequence is perhaps exhibited by infections of *P. floridense* in lizards. It also occurs in intense infections of *P. brasilianum* in monkeys. In less intense infections the merozoite mean does not vary more than 15% throughout the entire infection. In infections of *P. cathemerium* in canaries, *P. gallinaceum* in chickens, and *P. cynomolgi* and *P. knowlesi* in rhesus monkeys, the merozoite mean is high at first, decreases during the acute rise to a low point (30 to 60% of the original value) at or near the parasitological crisis and thereafter rises during the developed infection.

Moreover, the species may differ among themselves over a wide range with respect to the absolute number of parasites that are produced, and temporary decreases from 10 to 85% during infection have been repeatedly found for various malarias since the work of Boyd and Allen (1934) and especially Boyd (1939). The maximum number of merozoites per segmenter per day varies as follows: 3½ in *P. brasilianum*, 7½ in *P. cynomolgi* and in *P. lophurae*, 11 in *P. knowlesi* and 16 in *P. cathemerium* and *P. gallinaceum*. The species also differ in the absolute number of parasites that die (0 to 70% during innate immunity and 90 to 98% when acquired immunity is superimposed on innate immunity), the length of the acute rise (3 to 40 days) and the number of parasites at the peak (0.025 to 20 per 10 red cells or 10,000 to 6,000,000 per cmm blood).

We have ascribed the death of parasites and the decreases in the merozoite mean per segmenter to immunity as broadly defined. Immunity in this sense can be divided into two parts. Natural or innate immunity chiefly involves immunologically nonspecific factors which together lessen the suitability of the host as a culture medium for the parasite and is operative before as well as after acquired immunity supervenes. Acquired immunity involves most characteristically specific immunological mechanisms such as antibodies. It may also involve to a certain extent an in-

crease of nonspecific factors such as an increase in phagocytic cells. These may be defined as either innate or acquired immunity according to the authors' views. To us, the most prominent effect of acquired immunity is the precipitous drop in the number of parasites, i.e., the crisis, which terminates the acute rise of many initial infections and starts the so-called developed infection. That this effect is a fundamental phenomenon of acquired immunity is indicated by the fact that it is associated with the increased ability of the macrophages of the spleen, liver and bone marrow to ingest parasites and parasitized erythrocytes and can be duplicated histologically by the introduction of large numbers of parasites into latently infected animals (cf. Taliaferro and Cannon, 1936). The best evidence at present is that it is due to antibodies. In addition, the parasite decline terminating recurring relapses in treated infections of *P. knowlesi* can be shown to be associated with a high titer of protective antibody (Coggeshall and Kumm, 1938). Some recent workers have stressed the possible role of nonspecific factors, such as a decrease of mature red cells which are preferred by certain plasmodia, a rise in carbon dioxide in the blood (Rigdon and McCain, 1947) and the disappearance of infectible red cells as the infection progresses (Black, 1948). Without denying the role of such factors, it should be emphasized that acquired immunity persists for long periods during latent infections when most nonspecific factors have returned to normal (see Taliaferro, 1948c).

The lengthening of the asexual cycle, delay in the time of maximal segmentation, lowering of the merozoite mean and increase in the occurrence of crisis forms in knowlesi infections, all of which were more or less progressive, are reminiscent of changes in other malarias which are associated with crises and in our opinion of the development of acquired immunity. Previously, we (1944) concluded that similar phenomena in some infections with *P. brasilianum* are in part an expression of fundamentally parasiticidal antibodies and in part of nonspecific phenomena. We believe both sets of factors operate for infection with *P. cynomolgi* (1947) and *P. knowlesi*, but the extent to which each operates is not clear.

CONCLUSIONS

Blood-induced infections of *P. knowlesi* were studied in 18 rhesus monkeys with respect to the number of merozoites produced by segmenters at each asexual cycle, the length and synchronism of the asexual cycle and changes in parasitemia. The first two of these give the rate of reproduction of the parasites and in conjunction with parasite counts show survival or, conversely, death rates per segmentation.

Approximately 60% of the monkeys had progressively acute fatal infections, and the remainder had intermittently fatal ones. Many more large uninucleated and multinucleated stages disappeared from the blood during the first few segmentations of some patent infections than of others.

The asexual cycle was essentially 24 hours long. Maximal segmentation occurred around noon early in each infection over a period of 9 months. Although it was often gradually delayed and prolonged from one to several hours, it was occasionally accelerated several hours. Segmenters formed from 6 to 16 merozoites (mean of 10 to 11) early in each infection and formed 3 to 14 merozoites later (mean of 6 to 8, occasionally 4.5). The lengthening of the asexual cycle and decrease in the merozoite mean per segmenter indicate that the rate of reproduction was reduced to

50 to 75% (rarely to 40%) of its initial value.

The over-all death of parasites per segmentation was practically nil at the beginning of patent infections but increased to approximately 90% at the end of progressively acute infections. It was generally slightly higher in intermittently acute infections except for transient intermissions when it was practically nil or low.

The gradual and progressive lengthening and derangement of the asexual cycle, lowering of the merozoite mean, occurrence of degenerate-appearing crisis forms and increase in death rates indicate that some immunity is acquired by rhesus monkeys against knowlesi infections, even progressively acute ones.

REFERENCES

Aberle, S. D. 1945, Primate Malaria, Washington, D. C. National Research Council, pp. 171.

Afridi, M. K. 1938, J. Malaria Inst. India, **1**: 355–389.

Black, R. H. in Maegraith, B. 1948, Pathological Processes in Malaria and Blackwater Fever, Springfield, Illinois, Charles C Thomas, pp. 29–74.

Boyd, G. H. 1929a, Am. J. Hyg. **9**: 181–187.

———. 1929b, J. Exper. Zool. **54**: 111–125.

———. 1933, Am. J. Hyg. **18**: 295–323.

———. 1939, Am. J. Hyg. **29**(C): 199–129.

Boyd, G. H. and Allen, L. H. 1934, Am. J. Hyg. **20**: 73–83.

Brug, S. L. 1934, Riv. di malariol. **13** (9th new series, sect. 1): 121–142.

Cannon, P. R. 1941, in a Symposium on Human Malaria, Publication No. 15 of the Am. Assn. for the Advancement of Science, pp. 214–222.

Coggeshall, L. T. 1937, Am. J. Trop. Med. **17**: 605–617.

Coggeshall, L. T. and Eaton, M. D. 1938, J. Exper. Med. **68**: 29–38.

Coggeshall, L. T. and Kumm, H. W. 1937, J. Exper. Med. **66**: 177–190.

———. 1938, J. Exper. Med. **68**: 17–27.

Hartman, E. 1927, Am. J. Hyg. **7**: 407–432.

Jolly, A. M. D., Lavergne, and Tanguy, Y. 1937, Ann. Inst. Pasteur, **58**: 297–325.

Mulligan, H. W. 1935, Arch. Protistenk. **84**: 285–314.

Mulligan, H. W. and Sinton, J. A. 1933, Rec. Malaria Survey India, **3**: 529–568.

Reilly, J., Chen, G. and Geiling, E. M. K. 1949, J. Infect. Dis. **85**: In press.

Rigdon, R. H. and McCain, B. E. 1947, Am. J. Trop. Med. **27**: 673–681.

Simpson, M. L. 1948, Am. J. Hyg. **47**: 315–334.

Sinton, J. A. and Mulligan, H. W. 1933, Rec. Malaria Survey India, **3**: 357–443.

Stauber, L. A. 1939, J. Parasitol. **25**: 95–116.

Taliaferro, L. G. 1925, Am. J. Hyg. **5**: 742–789.

———. 1928, J. Prev. Med. **2**: 525–540.

Taliaferro, W. H. 1948a, Bact. Rev. **12**: 1–17.

———. 1948b, J. Infect. Dis. **83**: 164–180.

———. 1948c, Proc. Fourth Internation. Congresses Trop. Med. and Malaria, **1**: 776–782.

Taliaferro, W. H. and Cannon, P. R. 1936, J. Infect. Dis. **59**: 72–125.

Taliaferro, W. H. and Kelsey, F. E. 1948, J. Infect. Dis. **83**: 181–199.

Taliaferro, W. H. and Mulligan, H. W. 1937, Indian Med. Research Memoirs, No. **29**: pp. 138.

Taliaferro, W. H. and Taliaferro, L. G. 1934a, Am. J. Hyg. **20**: 1–49.

———. 1934b, Am. J. Hyg. **20**: 50–59.

———. 1944, J. Infect. Dis. **75**: 1–32.

———. 1947, J. Infect. Dis. **80**: 78–104.

———. 1948, J. Infect. Dis. **82**: 5–30.

———. 1949, J. Infect. Dis. **84**: 187–220.

Taliaferro, W. H., Taliaferro, L. G. and Simmons, E. L. 1945, J. Infect. Dis. **77**: 158–176.

Thompson, P. E. 1944, J. Infect. Dis. **75**: 138–149.

METABOLIC CHANGES IN ORAL LACTOBACILLI

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This study is a description of certain changes in nutritional requirements and metabolic behavior which accompany changes in the fermentation of mannitol, sorbitol and milk, described by Harrison[1,2] as occurring sporadically in certain cultures of oral lactobacilli. These changes consist in the occasional conversion of certain strains of lactobacilli from a state in which the culture does not ferment sorbitol or mannitol during two weeks' incubation in the sorbitol or mannitol broth described by Harrison[1] to a state in which the fermentation is manifested within three or four days. The fermentation of sorbitol and mannitol is frequently (always in the experiments described here) accompanied by the production of acid, coagulation and reduction in litmus milk. In this paper the term "fermenter" is used for those organisms which give the above reaction and the term "non-fermenter" for those organisms which do not.

METHODS

Cultures.—The four cultures used were two non-fermenting cultures, EH78 and EH41, and two fermenting cultures, 96A and 3A1S, from the collection of Harrison. These cultures were selected because their fermentation reactions, positive or negative, remained the same during monthly transfers in Jay's broth[3] for two years or more. Stock cultures were kept in Jay's broth or tomato juice yeast extract broth,[4] and in each experiment 0.1 ml of stock culture was transferred to tomato juice yeast extract broth and allowed to grow about 24 hours. The organisms were centrifuged down, washed three times with 0.85% sodium chloride and suspended in a volume of saline equal to the volume of medium in which they were grown. One-tenth ml inoculations from the suspension were usually made immediately or within 24 hours, except as otherwise stated. The mediums used in the fermentation tests were those described by Harrison.[1]

Synthetic medium.—The basal synthetic medium was that of Henderson and Snell,[5] containing salts, amino acids, purines, pyrimidines, vitamins and 1% glucose. The medium was modified in some of the experiments by doubling the amounts of *dl*-alanine, *l*-arginine, *dl*-aspartic acid, *l*-glutamic acid, *l*-histidine, *dl*-isoleucine, *dl*-methionine and *dl*-phenylalanine, and by replacing 20 μg of pyridoxal with 40 μg of pyridoxin per 100 ml of medium. We have no evidence that this modification is advantageous.

A modification of the basal medium contained 25 ml casein hydrolysate ("vitamin free," General Biochemicals, containing 12 mg nitrogen per ml) per liter and 100 mg tryptophane per liter as the only source of amino acids.

All modifications of the synthetic medium were adjusted to an initial pH of 6.5 to 7.0 and autoclaved 5 minutes at 20 pounds in tubes containing 4 ml of medium.

EXPERIMENTAL

The amino acids and vitamins essential for the organisms were determined by omitting constituents singly from the basal medium. All four organisms required alanine, arginine, aspartic acid, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methoinine, phenylalanine, threonine, tryptophane, tyrosine, valine, nicotinic and pantothenic acids, and biotin and riboflavin to greater or lesser degree. In addition to the above the fermenters required serine or tryptone but not thiamin. The

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1. Harrison, R. W. 1939, J. Infect. Dis. **65**: 142.
2. Harrison, R. W. 1942, J. Infect. Dis. **70**: 77.
3. Jay, P. 1927, Dental Cosmos, **69**: 977.
4. Weiss, J. E. and Rettger, L. F. 1934, J. Bact. **28**: 501.
5. Henderson, L. M. and Snell, E. S. 1948, J. Biol. Chem. **172**: 17.

non-fermenters required thiamin but not serine or tryptone.

In synthetic medium containing all the required amino acids for each strain, all four organisms attained maximum growth in 72 hours or less. At full growth the fermenters had produced the equivalent of about 8 ml of 0.1 *N* acid per 4 ml of medium (a yield of two moles of acid per mole of glucose), the non-fermenters considerably less,—the equivalent of about 5 ml of 0.1 *N* acid per 4 ml of medium. With good growth, the acid produced divided by the optical density gave a constant (average deviation from the mean, 4%) which was identical for both fermenters and about 80% higher than the constant which was identical for the non-fermenters. The final pH of the fermenter cultures was usually 3.5 ± 0.1 and of the non-fermenters, 4.0 ± 0.1. The amount of acid produced and the final pH were the same if the citrate of the medium was replaced by phosphate of equivalent buffering capacity.

Typical results are given in table 1. The synthetic medium contains the amino acids plus vitamins listed above as essential, except for the deficiencies noted. A deviation from the usual behavior was occasionally observed in the fermenting organisms and is illustrated by the two sets of figures, for organism 3A1S, which were obtained in simultaneous duplicate experiments with the same washed suspension and medium. These figures for 3A1S indicate the occurrence in one of the tubes lacking serine, but not in the duplicate, of organisms not requiring serine and showing delayed but considerable growth.

It was observed during the course of the above experiments that some cultures of the non-fermenters gave positive fermentation tests when tested in mannitol and sorbitol broths and litmus milk, and that these positive fermentation reactions were retained on repeated subculture in tomato juice yeast extract broth. The frequency of such changes was considerably higher than the frequency of the same change observed by Harrison, in whose experiments washed suspensions were not prepared. That this increase in frequency could be largely attributed to changes occurring in the washed suspensions was shown by comparing the fermentation reactions of suspensions with the fermentation reactions of the stock cultures and subcultures from which they were prepared, and by the experiment described in the next paragraph. The frequency of the change in the experiments described here varied considerably from week to week. The average frequency of change was 30% during a month in which about 100 tests for change were made. The reverse change (from a fermenting to a non-fermenting culture) was not observed in several hundred tests. It is to be noted, as will be pointed out later, that the fermentation tests are positive for and particularly sensitive for detecting fermenters. The results for the fermenter 3AlS in table 1 may indicate either that the suspension

TABLE 1.—*Effect of medium on growth.*

Organism	Duration of growth			Medium lacking in:
	17 hr.	24 hr.	72 hr.	
3A1S	0.022	0.025	0.051	Serine
3A1S	0.016	0.023	0.570	
96A	0.029	0.032	0.043	
EH78	0.627	0.627	0.727	
EH41	0.502	0.468	0.546	
3A1S	0.391	0.549	0.755	Thiamine
3A1S	0.371	0.539	0.710	
96A	0.397	0.463	0.577	
EH78	0.227	0.243	0.279	
EH41	0.157	0.143	0.190	
3A1S	0.023	0.031	0.069	Serine and thiamine
3A1S	0.017	0.028	0.058	
96A	0.022	0.029	0.067	
EH78	0.000	0.023	0.028	
EH41	0.099	0.093	0.091	

3A1S and 96A are "fermenters"; EH78 and EH41 are "non-fermenters." The numerical values are proportional to optical densities at 540 mμ.

contained a very small fraction of non-fermenters or that non-fermenters arose from fermenters during the course of the experiment. The change from non-fermenting to fermenting occurred with greater frequency in cultures derived from some suspensions than from others, and, provided the medium in which the change was first observed was adequate for the fermenting type of organism, no correlation appeared to exist between the composition of the medium and the frequency of change.

From a stock culture of a non-fermenter, 16 simultaneous subcultures were made to tomato juice yeast extract broth, and from these subcultures were made 16 suspensions which were kept in the refrigerator (except that 4 were kept in the incubator for the first 7 days of the experiment and subsequently in the refrigerator). At approximately three-day intervals, 0.1 ml of each suspension was inoculated into tomato juice yeast extract broth and into casein hydrolysate medium. After growth was established, fermentation tests were made. Only one fermenting culture occurred; this was in tomato juice yeast extract inoculated from a suspension which had stood in the refrigerator 14 days and had on three previous occasions given non-fermenting cultures. The total non-fermenting cultures were about 100 in number. Identical procedures in this experiment were carried out simultaneously as much as practical, and light more intense than diffuse daylight was avoided.

In another experiment designed to locate the part of the experiments in which the change from non-fermenter to fermenter took place, 12 sets of inoculations from stock cultures of the two non-fermenters were made into a total of 97 tubes of tomato juice yeast extract medium and 10 tubes of complete synthetic medium. The inoculations from both stock cultures were made at intervals of about a week. All the stocks at the time of inoculation and all the 107 tubes inoculated therefrom gave negative fermentation tests. Saline suspensions prepared as usual from the 107 transferred cultures all gave negative fermentation tests, with four exceptions, both immediately after preparation and at two- to three-day intervals during the two weeks in which the suspensions contained viable organisms. The four exceptions were suspensions prepared on the same day from tomato juice yeast extract cultures, three being from culture *a* of EH78 and one from culture *b* of EH41. Two other suspensions from culture *a* of EH78 and four other suspensions from culture *b* of EH41 gave negative fermentation reactions when tested on other days. The three suspensions from EH78 cultures showed trace fermentations in sorbitol and trace-to-complete fermentations in milk, both immediately after preparation of the suspension and on two and four days subsequently. On transfer of organisms from the test medium showing positive reactions to tomato juice yeast extract medium, cultures showing complete fermentation in milk, sorbitol and mannitol were obtained in every case. The single suspension of EH41 showing fermentation gave a strong fermentation in mannitol only, and organisms transferred from the mannitol test medium to tomato juice yeast extract medium showed complete fermentation in milk, sorbitol and mannitol mediums. Here, as in the previous experiment, the first demonstrable change in fermentation is seen in the suspended organisms. The coincidence of changes in the two organisms on the same day after repeated failures to change suggests an uncontrolled factor in the medium, technique or environment as being essential to the occurrence of the change.

In order to further describe the fermenting cultures which arose from non-fermenting cultures the following experiment was performed. Eight fermenting cultures (A through H) which had originated from non-fermenting cultures were used to prepare washed suspensions from each of which inoculations were made into 8 synthetic mediums. These mediums were of the same composition as the basal medium except that in medium #1 serine was omitted, #2 thiamin was omitted, #3 tryptone replaced thiamin and serine, #5 tryptone replaced serine, #6 tryptone replaced thiamin, #7 tryptone was added, #8 serine and thiamin were omitted. Medium #4 contained the basal medium. After 72 hours the growth was measured and the medium (4 ml per tube) titrated.

The suspension prepared from culture A gave little growth in medium #1 and #8 but maximal growth in the remaining tubes and maximal titers of 8 ml of 0.1 *N* alkali per tube. This behavior is identical with the pattern obtained in similar medium of suspensions from the original fermenting culture 96A and 3A1S. The experiment indicates that fermenters clearly predominated over non-fermenters in the suspension.

The suspension prepared from culture B gave maximal growth in all mediums except #8 and titration of 5 ml of 0.1 *N* alkali in medium #1, #2, #4, #5, #7 and titration of 7 ml of 0.1 *N* alkali in medium #3 and #6, indicating a predominance of non-fermenters in the suspension (table 2). The remaining suspension gave patterns of growth and acid production intermediate between the two cited above. In general there was a predominance of fermenters.

The quantitative significance of the fermentation reactions as a measure of the relative numbers of fermenting and non-fermenting organisms present in a culture was examined in the following experiment: Suspensions were made in the usual way from the fermenting cultures 96A and 3A1S and the non-fermenting cultures EH78 and EH41. Dilutions of the two suspensions from the fermenting cultures with saline were made as follows: 0.1, 0.01, 0.001, 0.0001, 0.00001 and 0.000001. Each diluted suspension was mixed with an equal volume of undiluted suspension derived from one of the non-fermenters to give four sets of suspensions, each set containing a different combination of a fermenter with non-fermenters (96A, EH78; 96A, EH41; 3A1S, EH78; 3A1S, EH41). One-tenth ml of each of the resulting mixtures was inoculated in duplicate into each of the three fermentation mediums. At the end of two weeks, complete fermentative reactions had taken place in all the fermentation tubes except one of the two duplicate tubes derived from one of the suspensions containing a 0.000001 dilution of the fermenter suspension. The experiment, repeated at greater dilution with two mixtures in which the suspen-

TABLE 2.—*Variation in changed cultures.*

Medium no.	Modification of basal medium	Culture A		Culture B	
		Growth	Titer (ml)	Growth	Titer (ml)
1	No serine	±	0	++++	5
2	No thiamin	++++	8	++++	5
3	No thiamin or serine; tryptone added	++++	8	++++	5
4	None	++++	8	++++	5
5	No serine; tryptone added	++++	8	++++	5
6	No thiamin; tryptone added	++++	8	++++	7
7	Tryptone added	++++	8	++++	5
8	No serine or thiamin	±	0	±	0

sion of non-fermenting organism was shown not to give positive fermentation reactions, showed that the dilution of the fermenter could not be greater than 1×10^6 and give consistently positive results.

DISCUSSION

The percentage of observed changes in these experiments varied widely from week to week. This fact probably indicates that the environment of the organisms during some phase of the experiments critical to the change was insufficiently controlled. The evidence that washing the organisms increases the rate of change and that the change can take place in washed organisms in saline suspension makes the type of adaptive mechanism postulated by Hinshelwood[6] seem unlikely in this case. One might imagine that some part of the genetic nucleic acid became less stable in the absence of one or more metabolites.

Rogosa et al[7] showed that of 250 strains of lactobacilli tested by them, all requiring thiamin were heterofermentative. This fact and the fact that our fermenters produce two moles of acid per mole of glucose and require no thiamin suggests that the change observed above is from a heterofermentative to a homofermentative type of metabolism. This possibility is being investigated.

The lower final pH of the changed organisms is of interest in regard to their pathogeneity. Among oral lactobacilli the group in which the fermenters are found are those most clearly associated with the lesions of dental caries.[1] Because of the shape of the pH solubility curve for dental enamel, a 0.5 pH change at a pH range below pH 5.0 must have a very large effect on the rate at which enamel is dissolved.

SUMMARY

Nutritional and metabolic changes in certain strains of oral lactobacilli accompanying the changes in fermentation reactions observed by Harrison are described. The changes include changes in the requirement for serine and for thiamin, changes in the amount of acid produced from glucose and changes in the final pH attained by cultures in synthetic mediums.

In these lactobacilli the fermentation tests in litmus milk, sorbitol broth and mannitol broth appear to detect one changed organism in 10^6 unchanged organisms.

6. Hinshelwood, C. N. 1946, The Chemical Kinetics of the Bacterial Cell, Oxford.
7. Rogosa, M., Tittsler, R. P. and Geib, D. S. 1947, J. Bact. **54**: 13.

FEEDING TESTS ON HUMAN VOLUNTEERS WITH ENTEROCOCCI AND TYRAMINE

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Alpha-type streptococci have been isolated from foods incriminated in outbreaks of food poisoning where no other agents could be ascribed as the cause. In all instances where the biochemical properties of these strains have been studied, they were found to be identical with those of enterococci. When grown in suitable medium and fed to human volunteers, illness has in many cases followed. As a rule, the symptoms have been mild and have been concerned principally with abdominal cramping and diarrhea, nausea and vomiting occurring less commonly. Outbreaks have been attributed to imported Albanian cheese, American cheddar-type cheese,[1] Vienna sausage,[2] beef croquettes,[3] cocoanut cream pie,[3] turkey dressing,[4] canned evaporated milk,[5] charlotte russe,[5] roast beef[5] and ham Bologna.[5] The number of outbreaks attributed to turkey or turkey products appears to be increasing. Many outbreaks of this type of food poisoning are not reported.

In view of the fact that enterococci have been associated with food poisoning outbreaks in man, it is essential that if a strain of *Streptococcus faecalis* were used as a starter in cheese, it should be tested for its food poisoning properties. A Cheddar cheese of excellent quality has been produced using a specific strain of *Str. faecalis* as a starter.[6] Therefore, experiments with human volunteers were carried out with the cheese starter strain as well as with three other strains isolated from foods incriminated in outbreaks of food poisoning. Since it had been previously demonstrated that enterococci decarboxylate tyrosine to form tyramine and the starter strain produced detectable amounts of tyramine in cheese,[7] it was decided to determine the effect of tyramine when fed to human volunteers.

MATERIALS AND METHODS

All of the cheeses were prepared from pasteurized milk. Batch 1L5487 was made May 7, 1948 from Cornell University milk to which 1% of Hansen's commercial lactic starter was added. Commercial starters usually consist chiefly of *Str. lactis* and *Str. cremoris*, together with small numbers of *Str. citrovorus* and *Str. paracitrovorus*. This cheese was fed without curing and was very low in the numbers of *Str. faecalis* and the amount of tyramine. Batch 4LDK5487 was made from the same lot of milk with 1% of Hansen's lactic starter and 1% of *Str. faecalis* cheese starter. This cheese was also fed without curing;

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This study was a cooperative project coordinated by Dr. G. M. Dack of the Food Research Institute of the University of Chicago, and aided by a grant from the National Cheese Institute. The cheese and starter cultures were furnished by Professor A. C. Dahlberg and Dr. F. V. Kosikowsky of the Department of Dairy Industry, Cornell University, Ithaca, New York. Dr. C. F. Niven, Jr. and Mr. A. G. Castellani of the American Meat Institute Foundation collected the cultures and were responsible for the bacteriological investigations. The clinical studies were carried out in the Department of Medicine of the University of Chicago by Dr. Joseph B. Kirsner and Dr. Homer Marshall.

1. Linden, B. A., Turner, W. R. and Thom, C. 1926, Pub. Health Rep. **41**: 1647–1652.
2. Cary, W. E., Dack, G. M. and Myers, E. 1931, Proc. Soc. Exper. Biol. & Med. **29**: 214–215.
3. Cary, W. E., Dack, G. M. and Davison, E. 1938, J. Infect. Dis. **62**: 88–91.
4. Dack, G. M. 1943, Food Poisoning, Chicago, University of Chicago Press.
5. Buchbinder, L., Osler, A. G. and Steffen, G. I. 1948, Pub. Health Rep. **63**: 109–118.
6. Dahlberg, A. C. and Kosikowsky, F. V. 1948, J. Dairy Sc. **31**: 275–284.
7. Dahlberg, A. C. and Kosikowsky, F. V. 1948, J. Dairy Sc. **31**: 293–303.

TABLE 1.—*Enterococci cultures.*

Number	Culture label as received	Received from:	Source or suspected food from which isolated	Remarks
R2B	*Str. faecalis* cheese starter	F. V. Kosikowsky, Cornell University	Human intestine	Experimental cheese starter.
R5	α-strep.-F.P. turkey dressing	G. M. Dack, University of Chicago	Turkey dressing	Reported to have originally produced food poisoning symptoms in human volunteers.[4]
R7	#1	Leon Buchbinder, New York City Department of Health	Spoiled canned evaporated milk	Reported to have produced experimental gastroenteritis.[5]
R26		Dr. L. V. Dieter, Los Angeles Department of Health	Turkey a la king	Eighty-nine persons ill May 24, 1948. Onset of symptoms ranged between 1½ to 15½ hours after eating, averaging 8½ to 9½ hours.

the numbers of *Str. faecalis* organisms were very high, but the tryamine content was low. The third batch of cheese, LDK34720, was made on March 20, 1947 with 1% of lactic and .1% of *Str. faecalis* cheese starter. The cheese was cured for 15 months at 60 F, so that at the time of the feeding experiments *Str. faecalis* had decreased to moderately small numbers, but the tyramine content was abnormally high—2700 gamma per gram of cheese. All of the cheese was made by the usual Cheddar process.

The history of the strains studied is given in table 1. The physiological characteristics of the experimental cheese starter, strain R2B, and those of three strains isolated from foods incriminated in food poisoning outbreaks are given in table 2.

Thirteen experiments were conducted with human volunteers, each experiment employing 4 volunteers. Thirty-seven subjects were fed, and of this number 15 were used again, making a total of 52 feedings. In many experiments 3 of the 4

TABLE 2.—*Physiological characteristics of cultures used in feeding tests.*

	Str. faecalis			*Str. liquefaciens*
	R2B	R5	R7	R26
Catalase	−	−	−	−
Nitrate reduction	−	−	−	−
Slime, sucrose gelatin agar	−	−	−	−
CO_2 from glucose	−	−	−	−
CO_2 from tyrosine	+	+	+	+
Growth:				
10 C	+	+	+	+
45 C	+	+	+	+
6.5% NaCl	+	+	+	+
325 units penicillin/liter	+	+	+	
40% bile blood agar	+	+	+	
Litmus milk	ACR	ACR	ACR	ACRP*
Litmus reduced before curdling	+	+	−	+
Blood agar	Wα†	α	α	Wα
Final pH, glucose	4.15	4.2	4.2	4.1
Hydrolysis:				
Starch	−	−	−	−
Sodium hippurate	+	SL‡	+	+
Esculin	+	+	+	+
Arginine	+	+	+	+
Gelatin	−	−	−	+
Fermentation:				
Xylose	−	+	+	−
Arabinose	−	+	+	−
Mannose	+	+	+	+
Fructose	+	+	+	+
Galactose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	+	+	+
Sucrose	+	+	+	+
Trehalose	+	+	+	+
Raffinose	+	SL	+	−
Inulin	−	−	−	−
Dextrin	+	+	+	+
Glycerol	+	SL	SL	+
Mannitol	+	+	+	+
Sorbitol	+	+	+	+

* ACRP—A, acid, C, curdled, R, reduction of litmus, P, proteolyzed.
† W—weak.
‡ SL—slight.

volunteers received the test samples; the 4th received a "blank" sample which contained few or none of the test bacteria. The sample which served as the control was known only to the laboratory workers. After preparing the samples they were turned over to the clinical investigators, who conducted the actual feeding tests. The tests were divided into three categories; namely, feeding of experimental cheeses, feeding of tyramine and feeding of cultures. The details and results of the tests are given in table 3 and do not follow the order in which the experiments were carried out.

At the beginning of the investigation an attempt was made to determine the enterococcus counts in the stools of the volunteers before and after the feeding tests to detect possible changes in numbers of enterococci. A modified form of White and Sherman medium[8] was used for quantitatively determining the numbers of enterococci present. This medium consisted of 0.5% glucose, 0.5% tryptone, 0.5% yeast extract, and 1.5% agar, with a reduced amount of sodium azide (0.01%) and a reduced amount of penicillin —not over 100 units per liter of medium. In this modified medium no quantitative inhibition of enterococci took place although colony sizes were smaller than those on control medium with no inhibitory substances added. With the exception of an occasional colony of a lactobacillus, no other types of bacteria which were encountered in the stool specimens would grow in this medium.

Stool specimens were collected from the volunteers usually just prior to the feeding tests and then again after 18 to 24 hours. The stool samples were kept in the refrigerator until tested, usually not over 8 hours after collection. One gram portions were placed in 99 ml of sterile water and emulsified in a mechanical blendor. Appropriate dilutions were plated on penicillin-azide agar. The results of these attempts to count enterococci in stool specimens are not given since it was concluded that they were meaningless for the following reasons: (1) extreme variations in the enterococcus counts of stools from one volunteer to another, (2) variations in counts in stools from one volunteer at different times, (3) inability to distinguish between enterococci experimentally fed and those existing normally in the intestine and (4) no knowledge as to whether the second stool specimen collected contained the enterococci which were fed.

The volunteers were young adults varying from 18 to 32 years of age. Four volunteers were hospitalized at a time in a 4-bed ward. The 32 volunteers in the first 7 experiments were hospitalized at noon the day before taking the test substance, ate two meals in the hospital and ate nothing during the test period but hospital food. The test substance was taken at 8 to 8:30 or 9 to 9:30 a.m. with breakfast. They remained in the hospital for the next 24 hours, eating only hospital food. Stool cultures were collected on the morning of the test before breakfast and again 24 hours later. Blood counts, sedimentation rates and urinalyses were done on admission and about 12 hours after taking the test substance. Temperature, pulse and respiration were recorded 4 times daily.

The 24 subjects in the last 6 experiments (8–13) were hospitalized and managed as were the 32 volunteers in the first 7 experiments, except that they were given the test substance at the evening meal of the first day and hospitalized for only 24 hours. No stool cultures were collected on this group, but other laboratory tests were done in the same manner as those of the first group. The volunteers were requested to report any abnormal symptoms that developed within two days after leaving the ward.

No abnormal changes were found in the red or white blood cell counts, the hemoglobin values of the blood or the sedimentation rates for blood cells. Examination of the urine for specific gravity, sugar, albumen, and the microscopic appearances of the sediment revealed no abnormalities. The temperature, pulse and respiration on all volunteers were within normal limits throughout the experiments.

DISCUSSION

The few illnesses occurring among volunteers consuming enormous numbers of enterococci are striking and raise many questions regarding this type of food poisoning. Perhaps the first question is how this work can be reconciled with naturally occurring outbreaks where a definite item of food containing millions of enterococci per gram is incriminated and a large number of people become ill. Is a specific menstruum or food necessary in this type of food poisoning? Do the strains change under conditions of cultivation in the laboratory so that they lose the ability to cause gastro-intestinal upsets when

8. White, J. C. and Sherman, J. M. 1944, J. Bact. **48:** 262.

TABLE 3.—*The effect of feeding human volunteers tyramine and enterococci grown in cheese, milk or meat medium.*

Experiment	Material fed	Total dosage—enterococci in billions, tyramine in mg	Subject	Age	Sex	Remarks
	Control: 100 g cheese prepared May 7 with commercial cheese starter (*Str. lactis*). Batch #1L5487. Fed May 24.	0.00016, 0.7 mg tyramine	1	18	M	
1.	100 g experimental cheese prepared from commercial cheese starter in combination with *Str. faecalis* cheese starter. Batch #4LDK5487. Fed May 24.	78, 2.3 mg tyramine	2 3 4	22 23 26	M M M	No effect.
2.	No control. 150 g cheese of Batch #4LDK5487 after cheese had remained at 34 C for 22 additional days. Tyramine content not determined. Fed June 15.	93	25	27	M	Had feeling of fullness for 4 hours after eating.
			26 27 28	31 28 29	M M M	Had nausea while eating and a feeling of fullness for 8 hours afterward. No diarrhea.
	Control: 100 g of Batch #1L5487.	0.00016	5	22	M	
3.	100 g of old, sharp, well-ripened cheese prepared from *Str. faecalis* starter. Batch #LDK34720. Fed May 26.	0.0004, 270 mg tyramine	6 7 8	23 30 26	M M M	No effect.
	Control: 1 pint freshly pasteurized milk.		9	28	M	
4.	30 ml of a 1% aqueous solution of tyramine monohydrochloride, C.P. (Pfanstiehl) were added to each of 3 pints of freshly pasteurized milk. (30 ml of milk were removed prior to adding the tyramine.) Fed June 1.	300 mg tyramine monohydrochloride	10 11 12	23 21 22	M M M	No change in blood pressure during 1½ hour observation period after feeding.
	Control: 1 pint pasteurized milk.		14	22	M	
5.	1 g tyramine monohydrochloride dissolved in each of 3 pints of freshly pasteurized milk. Fed June 3.	1000 mg tyramine monohydrochloride	13 15 16	25 32 24	M M M	No change in blood pressure during 1½ hour observation period after feeding.
	Control: 100 ml sterile beef heart infusion made up to 1 pint with milk		19	29	M	
6.	100 ml of the supernatant fluid from a 22-hour beef heart medium culture of *Str. faecalis* (R5, turkey dressing), each made up to 1 pint with pasteurized milk. Fed June 8.	40	17 18	27 24	M M	No effect.
			20	32	M	Mild flatulence.
	Control: 100 ml supernatant from sterile beef heart medium made up to 1 pint with milk.		24	21	F	
7.	100 ml of supernatant from 22-hour beef heart culture of *Str. faecalis* (R2B, cheese starter) made up to 1 pint with pasteurized milk. Fed June 10.	42	21 22 23	24 23 24	F F F	No effect.
	Control: 250 ml of commercially prepared cultured skimmed milk.		30	23	M	
8.	1 quart of freshly boiled milk in 2 L flask was cooled to room temperature and inoculated with 10 ml of a 24-hour culture of *Str. faecalis* (R7, spoiled evaporated milk). This milk culture was incubated at 37 C for 22 hours. 250 ml of the culture were fed to each subject.	317	29 31 32	22 19 28	M M M	No effect.
9.	No control. 20 ml of a 20-hour milk culture of *Str. faecalis* (R2B, cheese starter) were inoculated into 1 L of pasteurized milk previously warmed to 37 C. The milk was incubated at 37 C for 4½ hours, divided into 4 250 ml portions and chilled to approximately 15 C. 30 minutes later each portion was fed to 1 of 4 volunteers. Fed July 5.	222	15 33 5 6	32 22 22 23	M M M M	No effect.
10.	No control. 30 ml of a 20-hour milk culture (R2B, cheese starter) were inoculated into 1500 ml of pasteurized milk previously warmed to 37 C. The milk was incubated at 37 C for 4½ hours, after which it was divided into 4 300 ml portions and chilled to 15 C. It was fed to each of 4 volunteers on July 7.	120	34	25	M	Momentary cramp immediately after drinking and another 1 hour later.
			28 35 14	29 29 22	M M M	No effect.

TABLE 3.—*continued.*

Experiment	Material fed	Total dosage—enterococci in billions, tyramine in mg	Subject	Age	Sex	Remarks
11.	No control. 1 L of pasteurized milk at 37 C was inoculated with 50 ml of a 24-hour milk culture of *Str. faecalis* (R5, Turkey dressing). The milk was incubated at 37 C for 4½ hours, after which it was divided into 4 250 ml portions and chilled to approximately 15 C. It was fed to volunteers within ½ of an hour after removal from incubator. Fed July 9.	54	32	28	M	
			19	29	M	No effect.
			1	18	M	
			26	31	M	
12.	No control. 100 ml of an 18-hour milk culture of *Str. faecalis* (R7, spoiled evaporated milk) were inoculated into 1 L of pasteurized milk previously warmed to 37 C. The milk was incubated at 37 C for 4½ hours, after which it was divided into 4 portions of 250 ml and chilled to 15 C. They were fed to 4 volunteers within ½ hour after being removed from incubator on July 12.	65	22	23	F	
			23	24	F	No effect.
			24	21	F	
			36	24	F	
13.	No control. 1 L of milk autoclaved for 15 minutes at 15 pounds pressure, cooled to 37 C and inoculated with 100 ml of a 24-hour milk culture of *Str. liquefaciens* (R26, turkey à la king). The culture was incubated at 37 C for 7 hours. A soft, rennet-like curd was present after incubation. The culture was divided into 4 portions of 200 ml each, which were cooled to 15 C and fed to volunteers within 1 hour after incubation. Fed August 30.	182	30	23	M	Burning, lower abdominal pain and nausea 32 hours after taking culture. He immediately had 7 diarrheal stools. The symptoms ended 7 hours after onset, and patient felt well after that period. Patient had breakfast in hospital, no lunch. Evening meal, at home, consisted of steak, corn, pudding and small amount of wine.
			31	19	M	Had 4 loose stools beginning 36 hours after taking milk culture and 3 loose stools the following day. No nausea, abdominal pain or fever. Ordinarily has 1 bowel movement per day.
			35	29	M	No effect.
			37	31	M	Was awakened from sleep 60 hours after drinking test culture with nausea and diarrhea. Had 5 loose stools within 6 hours of onset of symptoms. Diarrhea stopped, nausea persisted for 12 hours from onset of symptoms. Patient took all-day final examination first day of symptoms.

fed to man? Are the illnesses due to something elaborated during growth of the enterococci?

The evidence available from naturally occurring outbreaks of food poisoning in which enterococci were implicated is fragmentary and incomplete. The offending foods may not represent uniform samples. For example, in the outbreak involving beef croquettes,[3] some of the croquettes may have been cooked in the deep fat for longer periods than others, thus destroying the enterococci. Therefore, some individuals failing to react may have eaten croquettes in which all enterococci were killed. Another example is the outbreak involving Vienna sausage,[2] where not all of the cans contained viable enterococci. A further example is the outbreak involving turkey dressing,[4] in which several turkeys were involved and some may have been cooked longer than others, thus destroying the enterococci. Other variables are time and temperature of storage of food before eating. These factors may vary among items of food prepared for a meal, thus appreciably affecting the numbers of bacteria in individual servings of food.

In spite of all of these limitations for naturally occurring outbreaks, it is

obvious that in these outbreaks a much larger proportion of people developed symptoms of food poisoning than in the group of volunteers reported in these experiments (see table 4). In the naturally occurring outbreaks listed in table 4, a careful laboratory examination of the suspected food items excluded salmonella, staphylococci and other bacterial agents as the cause of the illnesses. The long incubation period from the time of eating the meal to the time of onset of symptoms would further exclude staphylococcus enterotoxin. Although the onset in many individual cases in an outbreak of food poisoning involving enterococci may be short, the average incubation period is longer than the average $2\frac{1}{2}$ to 3 hour period characteristic of staphylococcal poisoning.

Possibly the type of food in which the organisms are ingested may be of significance. This is suggested in the work of Osler et al,[9] who reported illness in 1 out of 3 volunteers fed milk cultures, in 2 out of 12 fed custard cultures and in 4 out of 11 fed egg salad cultures. The symptoms in the volunteers receiving the milk culture were mild in comparison to those receiving the custard and egg salad cultures. Obviously the number of volunteers in their experiments was too few to permit drawing definite conclusions on this point.

The problem of whether or not strains change with reference to the ability to cause food poisoning by long cultivation in the laboratory is not solved. In the Vienna sausage outbreak,[2] a volunteer fed a beef heart culture prepared from a freshly isolated strain developed symptoms similar in severity to those of the persons made ill in the outbreak. In experiments with strains freshly isolated from beef croquettes,[3] human volunteers developed mild symptoms from swallowing living veal infusion broth cultures. Osler and associates[9] produced sypmtoms in volunteers fed strains which had been under cultivation for periods varying from two months to one year. In the experiments reported in this paper (table 3, exp. 13), mild symptoms were observed only in the case of milk cultures prepared from a strain which had been under laboratory cultivation for approximately three months.

No evidence has been produced to indicate that illnesses were caused by products liberated during the growth of enterococci in a food or medium. Filtrates of cultures, when fed to volunteers,[2,3] have in all instances failed to cause symptoms of food poisoning. Osler and associates[9] call attention to a

Table 4.—*Some characteristics of certain naturally occurring outbreaks of food poisoning associated with enterococci.*

Outbreak	Food implicated	Number persons studied			Onset of illness following meal (in hours)
		Ill	Not ill	Total	
1[2]	Vienna sausage	75	107	182	4–12
2[3]	Croquettes (beef)	117	91	208	3–18
3[4]	Turkey dressing	294	99	393	7–15
4[5]	Spoiled canned evaporated milk	74	87	161	2–7
5[6]	Charlotte russe	3	0	3	5½–12
6[6]	Barbecued beef	74	13	87	3–15
7[6]	Ham Bologna	9	0	9	3–6
8*	Turkey à la king	94	4	98	1½–15¼

* Personal communication from Dr. L. V. Dieter, Director of Laboratories, City of Los Angeles, California.

9. Osler, A. G., Buchbinder, L. and Steffen, G. I. 1948, Proc. Soc. Exper. Biol. & Med. **67**: 456–459.

report of Sherman et al,[10] suggesting the possibility that the toxic principle of *Str. faecalis* poisoning may be tyramine formed by the decarboxylation of tyrosine by this organism. All 4 strains used in these experiments decarboxylated tyrosine (table 2), but 0.3 and 1.0 g doses of tyramine monohydrochloride were without effect when fed to human volunteers (table 3, exp. 4 and 5). There is no evidence, therefore, that tyramine is toxic to man when taken by mouth in the doses indicated.

Osler et al[9] stress the fact that young enterococcus cultures are important in producing the experimental disease in man. They had no success with cultures incubated for 20 hours but were successful with cultures incubated 5 hours, since with the latter intestinal disturbances were produced in 6, or possibly 7, of 26 human volunteers. In the case of the outbreak with Vienna sausage,[2] the contaminated cans probably had contained the organisms for some weeks before they were consumed. Likewise, the spoiled canned evaporated milk referred to by Buchbinder and associates[5] in all probability did not represent a young culture of the enterococci. In the experiments reported in this paper, cultures R2B, R5 and R7, grown in pasteurized milk for $4\frac{1}{4}$ or $4\frac{1}{2}$ hours at 37 C, were without effect when fed to human volunteers (table 3, exp. 9, 10, 11 and 12).

Buchbinder and associates[5] stress the point that very large numbers of enterococci may be necessary to produce gastro-intestinal symptoms. The present experiments do not seem to bear out this point, since dosages of from 40 to 370 billion viable enterococci have been fed without causing ill effects.

10. Sherman, J. M., Gunsalus, I. C. and Bellamy, W. D., 57th Annual Report, New York State College of Agriculture, Cornell University.

The striking point in this investigation was that no illnesses occurred in human volunteers fed large numbers of *Str. faecalis*. In experiment 2, where 150 g of cheese were given each of 4 volunteers, the symptoms of fullness and nausea were attributed to the large amounts of cheese eaten. Otherwise no illnesses resulted, except in experiment 13, involving *Str. liquefaciens*, where 3 of the 4 volunteers became ill some time after drinking milk cultures containing 182 billion cells of strains R26. It is questionable whether the illness of one of the three subjects (37) was related to the feeding. This subject was prone to develop gastro-intestinal disturbances when emotionally upset at the time of examinations. The fact that 60 hours elapsed from the time of taking the test sample would cast further doubt on the relation of the illness to the test feeding. In the case of volunteers 30 and 31, who became ill 32 and 36 hours after feeding, there was no explanation for their illnesses other than the test milk culture which they were fed. The symptoms were characteristic of food poisoning reported for enterococci except for the unusually long time after eating until the onset of symptoms.

SUMMARY

Experiments involving 52 feeding tests on 37 volunteers were carried out with the object of determining the safety of using a specific strain of *Streptococcus faecalis* as a starter culture for cheese. Since strains of *Str. faecalis* have been associated with food poisoning outbreaks and since they have been shown to decarboxylate tyrosine to produce tyramine, it was necessary to feed cheese made with the starter strain as well as cultures of the starter strain and, in addition, to determine whether tyramine may play a role in food poisoning. Cultures from three strains of entero-

cocci implicated in outbreaks of food poisoning were fed to human volunteers for control purposes.

Cheese made with the starter strain of *Str. faecalis* and which contained large numbers of viable organisms as well as appreciable quantities of tyramine was without effect when fed to human volunteers. Tyramine monohydrochloride, in 0.3 or 1 g amounts, when fed to human volunteers in one pint of milk, caused no rise in blood pressure or any other ill effects.

Illness occurred in two and possibly a third volunteer fed a milk culture of a strain of *Str. liquefaciens* recently isolated from an outbreak of food poisoning. *Str. faecalis*, in two strains from other outbreaks, was without effect when similarly fed to human volunteers. A discussion of the status of our knowledge of food poisoning caused by enterococci is detailed in the paper.

COMPLEMENT FIXATION WITH FECAL ANTIGENS IN VIRAL HEPATITIS

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Although attempts to develop a specific immunologic test for viral hepatitis have been unsuccessful, certain serologic responses have been observed in patients with the disease. Agglutinins for sheep erythrocytes,[1] and positive Wassermann and Kahn tests[2,3] have been described. Precipitins[4,5] and complement-fixing antibodies[1,6,7] have also been demonstrated in convalescent phase serums which react with antigens present in acute phase serums and in saline extracts of normal liver. Reports of the frequency of occurrence of such positive reactions have varied widely, and certain workers[8-10] have failed to substantiate the positive results of others.

An analogy has been made between the serum antigen-antibody system in viral hepatitis and a similar reaction between acute and convalescent phase serum in yellow fever.[1] Although the exact mechanism of this reaction is not clearly understood, it was originally suggested by Hughes[11] that the antibody elaborated in convalescence was a response to an antigenic substance resulting from injury to tissue and unrelated to any antigenic component of the causative virus. It is not known whether a similar mechanism is operative in viral hepatitis, although preliminary observations by Eaton et al[1] suggested that the serum antigen-antibody system is distinct from another reaction in which a heterogenetic antibody is concerned. These workers[1] determined the presence of a heterogenetic antibody in the serum of certain patients with viral hepatitis which agglutinates sheep erythrocytes and fixes complement with antigen made from saline extracts of normal human liver. Precipitins for an apparently similar antigen have also been detected in convalescent phase serum.[5] All of these reactions appear to be non-specific and of no practical value.

An inherent difficulty in the development of a specific serologic test has been

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1. Eaton, M. D., Murphy, W. D. and Hanford, V. L. 1944, J. Exper. Med. **79:** 539.
2. Kuzell, W. C. and Puccinelli, V., 1944, Bull. U. S. Army Med. Dept. **80:** 3.
3. Waelsch, J. H. 1946, Brit. M. J. **1:** 353.
4. Sawyer, W. A., Meyer, K. F., Eaton, M. D., Bauer, J. H., Putnam, P. and Schwentker, F. F. 1944, Am. J. Hyg. **39:** 337; **40:** 35.
5. Olitzki, L. and Bernkopf, H. 1945, J. Infect. Dis. **77:** 60.
6. Miles, J. A. R. 1946, Brit. J. Exper. Path. **27:** 25.
7. Findlay, G. M., Martin, N. H. and Mitchell, J. B. 1944, Lancet, **2:** 301, 340, 365.
8. Fox, J. P., Manso, C., Penna, H. A. and Pará, M. 1942, Am. J. Hyg. **36:** 68.
9. Oliphant, J. W. 1944, Pub. Health Rep. **59:** 1614.
10. Havens, W. P., Jr., Gambescia, J. M. and Knowlton, M. 1948, Proc. Soc. Exper. Biol. & Med. **67:** 437.
11. Hughes, T. P. 1933, J. Immunol. **25:** 275.

the apparent failure to transmit hepatitis virus to laboratory animals or embryonating eggs. It has not yet been possible to obtain an adequate amount of material containing sufficient virus to be demonstrably antigenic. Recent reports of the concentration and purification of influenza and poliomyelitis viruses by ultracentrifugation[12,13,21] and also by precipitation with methanol at low temperatures[14,15] suggested the possibility of concentrating hepatitis virus in materials presumed to be infectious. Since virus is known to be present in the feces of patients with infectious hepatitis during the acute phase of disease, it was hoped that virus might be purified and concentrated from such material by these methods to a degree sufficient to make an effective antigen.

Feces were obtained from patients with acute hepatitis in a United States military hospital in Germany. It is probable that many of these patients had homologous serum jaundice, suggesting the possibility that virus was not present in certain of the specimens. However, since there is no method of differentiating between this form of hepatitis and the naturally occurring disease, material from 7 to 14 patients was pooled in an attempt to obviate this difficulty.

Early in the course of experiments, a single attempt to induce hepatitis in volunteers by feeding an antigen prepared by precipitation with methanol and ultracentrifugation was unsuccessful, suggesting that it was not infectious.* Whether this indicated that virus had been destroyed by the process of concentration is not known, since the lack of volunteers had made it impossible to test the original feces for the presence of virus before the antigen was made. Although infectivity was not demonstrated, it was observed that this antigen and other similar ones made from feces of patients with hepatitis and also of normal persons had the capacity to fix complement in the presence of serums of patients with viral hepatitis. It is the object of this paper to describe the results of these tests.

METHODS AND MATERIALS

Serum.—One hundred and sixty-five specimens of serum were obtained from 63 patients with acute hepatitis in a United States military hospital in Germany. It is probable that many of these patients had homologous serum jaundice, but since there is no method of differentiating between this form of hepatitis and the naturally occurring disease, they are all classified as viral hepatitis. Serum was obtained from 56 patients in the second week of disease. Single or serial specimens at intervals of 7 to 14 days were obtained from 29 of these patients during convalescence. From 7 other patients the initial serum was obtained in the third week of disease, and subsequent specimens were obtained in convalescence. The serum was frozen at once and stored at a temperature of −4 C for periods ranging from 1 to 4 weeks. Subsequently, it was shipped to the United States in containers with dry ice and stored at dry ice box temperature for periods of 1 to 9 months before use. The normal serum used for control purposes was obtained from donors of blood at a blood bank and was stored at dry ice box temperature until used.

Serum was heated to a temperature of 60 C for 30 minutes immediately before use. A number of jaundiced serums were anti-complementary, and it was found that they usually could be made usable by centrifuging at a speed of 18,000 r.p.m. in an angle-head centrifuge for 10 to 20 minutes.

Antigens for complement fixation.—Preliminary experiments revealed that a large percentage of serums from patients with hepatitis fixed complement with antigens made from feces of normal persons as well as from feces of patients with

12. Stanley, W. M. 1945, J. Exper. Med. **81**: 193.
13. Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D. and Beard, J. W. 1945, J. Immunol. **50**: 291.
14. Cox, H. R., Van der Scheer, J., Aiston, S. and Bohnel, E. 1947, J. Immunol. **56**: 149.
15. Brumfield, H. P., Stulberg, C. S. and Halvorson, H. O. 1948, Proc. Soc. Exper. Biol. & Med. **68**: 410.

* This experiment was done by Dr. Joseph Stokes, Jr., Philadelphia, Pa.

hepatitis, suggesting that antigenicity was not related to infectivity. For this reason, a number of fecal antigens from normal persons and patients with the disease were made and tested in the experiments to be described.

Feces from normal persons and patients in the acute phase of viral hepatitis (10th to 14th day) were collected and frozen at −4 C for periods of 2 to 4 weeks. Subsequently, they were sent to the United States in containers with dry ice and stored at dry ice box temperature for periods ranging from one to two months before use. For the preparation of antigens, a 20% suspension was made of feces in isotonic solution of sodium chloride in a Waring Blendor. The suspension was filtered through layers of gauze to remove large particles and the filtrate spun at about 20,000 r.p.m. for 20 minutes in the Sharples centrifuge equipped with a cooling coil. The supernate of this was chilled to 0 C, and methanol, precooled to about −40 C, was added at a rate not exceeding 2 ml/minute in amount to equal 35% of the total volume. The mixture was agitated constantly by a mechanical stirrer at a rate of 500 to 1000 r.p.m. The temperature of the mixture was kept between −2 and 0 C. When all the methanol was added, the pH was adjusted to 4.5. The mixture was allowed to stand at least one hour and then was centrifuged at 2300 r.p.m. for 15 to 30 minutes at a temperature of −2 to 0 C. The supernate was discarded. The sediment was resuspended in 1/10 of the original volume of *M*/10 sodium phosphate buffer at pH 7.5. This was centrifuged in 250 ml containers at 2300 r.p.m. for 30 minutes at −2 to 0 C and the sediment discarded. Methanol was added to the supernate in amount of 35% of the total volume, and the pH was again adjusted to 4.5. The suspension was centrifuged at 2300 r.p.m. for 30 minutes and the supernate discarded. The sediment was resuspended in 1/10 the preceding volume of *M*/10 sodium phosphate, and the pH adjusted to 7.5. This was centrifuged at −2 to 0 C at 18,000 r.p.m. for one hour and the sediment discarded. The supernate was spun in a precooled rotor in an air-driven vacuum ultracentrifuge at 39,000 r.p.m. for one hour. The supernate of this was usually crystal clear and was used as antigen. The sediment was taken up in 1/10 of the original volume of *M*/10 sodium phosphate buffer at pH 7.5, centrifuged at 18,000 r.p.m. for 15 minutes, and the sediment discarded. The supernate was frequently thick and viscous and anticomplementary, reacting positively with a large percentage of serums from normal persons as well as from patients with hepatitis. For these reasons, only occasional antigens prepared from the resuspended sediment obtained from ultracentrifugation were satisfactory. One such preparation from feces of patients with hepatitis, which was an effective antigen, was apparently not infectious when fed to volunteers. Because of the apparent failure to detect virus in the sediment following ultracentrifugation, the difficulty in rendering such material fit for use, and the reactive capacity of feces from both normal persons and patients with hepatitis, it was decided to use the supernate of the ultracentrifuged material as antigen. All antigens were shell frozen in a mixture of dry ice and alcohol, lyophilized, and extracted twice at icebox temperature with 2½ volumes of benzene, for periods ranging from two hours to overnight. All traces of benzene were removed by vacuum pump and the antigen rehydrated in isotonic solution of sodium chloride. It was then filtered through a Seitz filter and tested for anti-complementary activity before use. Amounts of antigen usually required for one day's test were placed in tubes and preserved at dry icebox temperature.

Technique of complement fixation test.—The test was performed as follows: Two-tenths cc of inactivated serum in a dilution of 1:2 was mixed with 0.2 cc of serial twofold dilutions of antigen ranging from 1:4 to 1:32. To the mixture of antigen and antibody were added 2 full units of guinea pig complement contained in 0.25 cc. Complement was titered in the presence of the lowest dilution of antigen before each test. Appropriate anti-complementary and hemolytic control tests were made of each serum and antigen in each test. Normal serums were always tested along with serums from patients with hepatitis.

All tests were incubated in a water bath at 37 C for 2½ hours. The hemolytic system, consisting of equal parts of 3% sheep erythrocytes and amboceptor diluted to contain 2 units in 0.25 cc, was added to each tube in an amount of 0.5 cc. The tests were then incubated for 30 minutes at 37 C, after which readings were made. The titer of positive tests was recorded as the greatest dilution of antigen with which there was 50% fixation of complement. The frequency of positive tests with normal serums and serums of patients with hepatitis when antigens were used in dilution of 1:4 or less necessitated the use of a dilution of 1:8 of antigen as the minimum critical dilution.

Absorption experiments.—Efforts were made to determine the absorption capacity of human liver, washed sheep and rabbit erythrocytes, boiled guinea pig kidney, and boiled bovine erythrocytes for the antibody in hepatitis serums and normal serums. After absorption with these materials, the serums were inactivated at 60 C for ½ hour and diluted 1:2 for testing.

RESULTS

Single or serial specimens of serum from 63 patients in different phases of viral hepatitis were tested by complement fixation with various antigens prepared from feces of patients with the disease and from normal persons. Both types of antigens possessed widely divergent capacities to fix complement with serums from patients with hepatitis. A similar variation in reactivity was observed with normal serums, although the percentage of positive normal serums was significantly less with each antigen tested. In table 1 are recorded the results of testing certain serums and antigens, illustrating these variations.

TABLE 1.—*Capacity of 7 fecal antigens to fix complement with serums from patients with viral hepatitis and from normal persons.*

Antigen	Hepatitis serums		Normal serums
	Acute*	Convalescent	
SN-B 11 (H)	16/19	22/42	2/12
SN-B 16 (N)	29/40	72/92	14/36
SN-B 19 (H)	15/30	28/70	6/40
SN-B 12 (H)	8/18	13/32	2/14
SN-B 14 (N)	10/27	17/38	2/14
SN-B 20 (N)			11/39
SN-B 43 (N)			21/27

* Acute phase includes period up to 14th day of disease.
Numerator = number of tests positive.
Denominator = number of tests done.
(H) = Hepatitis.
(N) = Normal.

Because of the variability of response of serums with different antigens, a comparison was made between those antigens which were most reactive with serum of patients with hepatitis and least reactive with normal serum. When such antigens were employed (either normal or hepatitis), positive results were obtained in 52 out of 63 patients (82.5%) at some time during the course of disease. Only 15 out of 75 (20%) normal serums fixed complement with these antigens.

Relationship of positive tests to stages of disease.—Forty out of 56 patients had positive tests in the second week of disease, and 30 out of 36 patients were positive at some time during convalescence. Thirty-six patients were tested early in the course of disease (7th to 18th day), and again once or more later in the course of disease (19th to 76th day). In these patients, no definite trend of antibody response was observed. Positive titers of antigen ranged from 1:8 to 1:32. Sixteen patients had a demonstrable rise in titer during convalescence, 8 patients had a fall in titer from the initial acute phase level, and 9 patients manifested no change. Three patients were negative throughout the period of observation.

Although no consistent quantitative trend of antibody response was apparent, the number of serums reacting positively varied with the phase of disease. One hundred and sixty-five specimens of serum from 63 patients throughout the course of disease were tested, and the results are recorded in table 2. It is evident that a large percentage of patients (71%) have a substance in their blood capable of reacting with fecal antigens during the second week of disease. The number reacting positively increases during the 3rd and 4th weeks, diminishes slightly from the 5th through the 7th week, and declines still more from the 7th to the 12th week when, however, 13 out of 22 serums (59%) tested still were positive.

TABLE 2.—*Relationship of positive complement fixation tests to stage of disease in 63 patients with viral hepatitis.*

Days of disease	Number of serums		
	Tested	Positive	Percent
7–13	56	40	71
14–30	46	41	89
31–50	41	35	85
51–80	22	13	59

Complement fixation with fecal antigens by serums from patients with other diseases.—Single or paired specimens of

serums from patients with atypical pneumonia, cirrhosis of the liver, and from persons with positive Wassermann reactions were tested by complement fixation with fecal antigens which gave positive tests with serums from patients with hepatitis. Fifty percent of a small group of patients with hepatic cirrhosis had positive tests, but a much lesser proportion of positive reactions were found in the serums of persons with atypical pneumonia and with positive Wassermann tests. In table 3 the results are compared with the results of testing the same antigens with the serums of patients with hepatitis and normal persons.

Absence of the relation of antibody to fecal antigens to the principal human blood groups.—There is no reason to assume that the reagent in the fecal antigen is blood group substance, since such materials are destroyed in the lower intestinal tract.[16] Determination of blood group was done on the 63 patients with hepatitis, however (table 4), and the results arranged according to complement fixation reaction with fecal antigens (table 5). Because of the small numbers involved, no significance can be attached to the deflection from normal distribution, particularly in the percentages of groups A and O. Determination of blood groups on a larger number of patients with hepatitis by Eaton et al[1] and others[17] revealed an essentially normal distribution in this disease. It is evident that serums of all blood groups reacted almost equally well with fecal antigens (table 5). The positive response of 3 out of 4 patients of group AB adds further support to the concept that blood group substances are not involved in the reaction. In addition, there was no consistent pattern in the response of serums of the same blood group with a given antigen, and it was not uncommon to find an antigen reacting positively with one serum and negatively with another serum of the same blood group.

Absorption experiments.—Serums which reacted positively with a titer 1:32 of antigen were absorbed with unheated human liver, boiled guinea pig kidney, boiled beef erythrocytes, and unheated sheep and rabbit erythrocytes. None of the serums tested revealed any reduction in capacity to fix complement with the same antigens used before absorption.

Nature of the reacting antigen.—Heating to 60 C for 30 minutes, as well as

TABLE 3.—*Complement fixation reactions with fecal antigens and serums from patients with viral hepatitis and other diseases and from normal persons.*

Disease	Number of patients		
	Tested	Positive	Percent
Viral hepatitis	63	52	82.5
Hepatic cirrhosis	18	9	50.0
Atypical pneumonia	12	3	25.0
Positive Wassermann	24	3	12.5
Normal	75	15	20.0

TABLE 4.—*Distribution of blood groups in 63 patients with viral hepatitis.*

Blood group	Patients	
	Number	Percent
A	42	66.60
O	15	23.80
AB	4	6.40
B	2	3.20
Total	63	100.00

TABLE 5.—*Relationship of positive complement fixation tests to various blood groups in 63 patients with viral hepatitis.*

Blood group	Acute phase		Convalescent phase	
	Number patients	Positive tests	Number patients	Positive tests
A	37	25	19	15
O	13	10	12	11
AB and B	6	5	5	4
Total	56	40	36	30

16. Witebsky, E. and Neter, E. 1935, J. Exper. Med. **62**: 589.
17. Swift, W. E., Jr. Personal communication.

repeated freezing and thawing in dry ice-alcohol mixture, did not affect the antigenicity of the fecal materials. Extraction of lyophilized antigen with alcohol, ether, and benzene did not remove the reacting substance.

DISCUSSION

The data presented in this paper indicate that there exists in the serum of patients with viral hepatitis and, to a significantly lesser degree, in the serum of normal persons, a reagin capable of fixing complement in the presence of antigens prepared from feces of both normal persons and patients with hepatitis. Similar positive reactions were found in a goodly percentage of patients with hepatic cirrhosis and in a smaller percentage of patients with atypical pneumonia and persons with positive Wassermann tests. Although the exact nature of the reagin which reacts with fecal antigens is not known, it would seem that it may be placed in the category of heterogenetic antibody. A single attempt to demonstrate hepatitis virus in a reactive antigen prepared from the feces of patients with the disease was unsuccessful; however, the reactive capacity of fecal antigens made from both normal persons and patients with hepatitis suggested that the serologic response was not specifically related to the presence of hepatitis virus. The antigen is stable at 60 C for 30 minutes and is apparently insoluble in alcohol, ether, and benzene. The antibody is not absorbed by human liver, boiled guinea pig kidney or beef erythrocytes sheep and rabbit erythrocytes.

The antibody to fecal antigens in acute hepatitis can be distinguished from the antibody to human liver in this disease, described by Eaton et al,[1] by the fact that it was not absorbed by human liver, boiled beef erythrocytes, or rabbit erythrocytes. Failure of absorption with sheep erythrocytes and the absence of agglutinins for sheep cells in the serum differentiated it from the antibody of infectious mononucleosis. The fact that blood group substances are destroyed in the lower intestinal tract indicates that they are not components of the antigen. In addition, the distribution of principal blood groups among serums showing positive tests with fecal antigens revealed an essentially equal reactive capacity. Although certain Wassermann-positive serums also gave positive reactions with fecal antigens, none of the hepatitis or normal serums which reacted with the fecal antigens gave positive Wassermann reactions, suggesting that the positive response in these serums was not due to the Wassermann reagin.

Five out of 52 patients with complement-fixing antibodies to fecal antigens had positive Kahn tests. In three of them the titer was 10 Kahn units or less, while the other two had 144 and 320 Kahn units, respectively. Another patient who had no demonstrable complement-fixing antibody to any fecal antigen tested did have a positive Kahn titer of less than 10 units. All of these positive Kahn tests became negative within three months.

The possibility that products of bacteria inhabiting the intestinal tract are important components of such antigens must be considered. It has been reported that occasional patients with viral hepatitis who had been previously vaccinated against salmonella developed a moderate rise in titer of agglutinins to certain strains of those organisms during the course of hepatitis. This has been interpreted as an anamnestic reaction.[18,19] Whether patients with hepatitis undergo immunologic alteration of

18. Nichols, H. J. 1923, J.A.M.A. **81**: 1946.
19. Havens, W. P., Jr. and Wenner, H. A. 1946, J. Clin. Investigation, **25**: 45.

such a kind as to make possible the serologic responses described here is unknown. It is of interest that 50% of a small number of patients with hepatic cirrhosis had positive tests with fecal antigens. Partition of the serum proteins of these patients revealed a low serum albumin and an increased serum globulin, with reversal of the albumin/globulin ratio. Artificial alteration of the serum proteins of such patients by the parenteral administration of homologous albumin resulted in previously positive serums becoming negative, suggesting that in hepatic cirrhosis the positive complement fixation reaction with fecal antigens is, in part, dependent on the amount and ratio of serum proteins. Although patients with viral hepatitis have a consistent pattern of alteration in serum proteins during the course of disease, with early decrease in albumin and increase in globulin,[20] insufficient amounts of serum made it impossible to determine if this pattern of alteration reflects the changing capacity to react positively with fecal antigens in complement fixation.

20. Havens, W. P., Jr. and Williams, T. L. 1948, J. Clin. Investigation, **27**: 340.
21. Melnick, J. L. 1943, J. Exper. Med. **77**: 195.

SUMMARY

A reagin which fixed complement with antigens made from feces of patients with hepatitis and normal persons was found in the blood of certain patients with viral hepatitis.

The antigen concerned in this reaction was prepared by precipitation of extracts of feces with 35% methanol at pH 4.5, and further refined by sedimentation of interfering substances in the ultracentrifuge at 39,000 r.p.m. for one hour. It was stable at 60 C for 30 minutes and insoluble in alcohol, ether, and benzene.

The exact nature of the antibody is undetermined although it is suggested that it may fall in the category of heterogenetic antibodies.

Differences from certain known heterogenetic antigen-antibody systems were determined.

TOXEMIA AS A STIMULATION FACTOR IN THE PRODUCTION OF ANTIBODIES.

STUDIES ON PRECIPITIN PRODUCTION IN THE DOG.

I. THE USE OF EGG WHITE

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Not all species of animals readily produce precipitins when injected with a protein of large molecular size and of known antigenicity as in the rabbit. The horse, dog, ox and the human species belong to the class in which precipitin production is the exception rather than the rule. In the human, precipitins[1] for horse serum proteins have been found following the injection of diphtheria antitoxin. The percentage of cases showing any precipitins was relatively low and of these the number having reasonably high titers was very limited. In a series of 30 cases recovered from typhoid fever, all showed precipitins in relatively high titer for typhoid protein.[2] In these cases the disease produced a condition of toxemia, especially in the typhoid fever. Burky[3] found that rabbits could produce antibodies for the lens protein of the eye if he injected staphylococcal toxin and the antigen.

In the field of biological chemistry, precipitins have been produced in the rabbit for the protein of rabbit skin and lung with the aid of staphylococcal toxin prepared by Burky's method.[3] From these observations it seems probable that toxemia plays a role in the formation of precipitins.

If a satisfactory method could be found to stimulate precipitin formation in species that normally do not produce these antibodies and if such a method could be made applicable to man, it would aid in combating certain diseases.

This work was undertaken to attempt the production of precipitins in the dog. No precipitins are formed when a pure antigen is injected in the dog by the usual intravenous method of immunization. When the antigen is adsorbed on aluminum cream according to the method of Hektoen and Welker[4] and injected intramuscularly, no precipitins are formed. The attempt to stimulate precipitin formation by the intramuscular injection of the antigen adsorbed on aluminum cream and intravenous injection of staphylococcal toxin prepared according to the method of Burky[3] also gave negative results. Egg white diluted to 1% protein content with 0.9% sodium chloride solution injected into the dog by the intravenous route produces toxemia. In medium-sized dogs, 100 cc of a 1% solution of egg white injected intravenously at three-day intervals will kill the animals with the second or third injection. A dose of 50 cc was also found to be excessive. If the egg white solution is injected intravenously and at the same time an antigen, such as ox albumin adsorbed on aluminum cream according to the method of Hektoen and Welker,[4] is injected into the thigh muscles of a dog, antibodies appear for both the proteins of egg

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1. Wells, C. W. 1915, J. Infect. Dis. **16**: 63–71.
2. Spinka, I. M. and Mann, L. S. Personal communication.
3. Burky, E. L. 1934, J. Allergy, **5**: 466–475.
4. Hektoen, L. and Welker, W. H. 1933, J. Infect. Dis. **53**: 309–311.

white and for the antigen injected intramuscularly.

Ox albumin was prepared by the ammonium sulfate precipitation method. A 1% solution of this ox albumin was prepared with physiological saline solution. A volume and one-half of aluminum hydroxide cream prepared according to the methods of Marshall and Welker[5] and Tracy and Welker[6] was added to the ox albumin solution. This was then thoroughly agitated and centrifuged. The supernatant liquid was discarded. The remaining material was then made up to the original volume of the ox albumin solution with physiological saline solution. This suspension was then stirred until it had the proper consistency suitable for use in a syringe. This suspension was designated ox albumin adsorbed on aluminum hydroxide cream.

A 1% egg white solution was prepared by diluting egg white (which had been passed through cheese cloth and thoroughly agitated) with physiological saline solution until the protein content was 1%.

EXPERIMENTAL

In the first experiment 50 cc of ox albumin adsorbed on aluminum hydroxide cream was injected into the thigh muscles of a medium-sized dog. The injection of the ox albumin adsorbed on aluminum hydroxide cream must be distributed in small depots in the muscles to avoid pressure necrosis. Three days later 25 cc of a 1% egg white solution was injected intravenously. Two weeks after the last injection of egg white, the blood serum of this animal showed a precipitin titer of 1:10,000 for ox albumin and 1:100,000 for egg white. The precipitin test was run by the layer method with solutions of the antigens in various dilutions.

In the second experiment 5 dogs were used (#22, 76, 52, 75 and 70). Each animal was injected with 50 cc of ox albumin adsorbed on aluminum hydroxide cream into the thigh muscles. In 4 of the animals 30 cc of egg white solution was injected intravenously. The 5th dog (#76), which was slightly heavier, was given a dose of 35 cc. Similar injections of the egg white solution were repeated on the 4th day. Ten days after the last injection of egg albumin solution, the blood serums were tested for the precipitin titer to ox albumin, egg white solution, fowl albumin, egg albumin, ovomucin and ovomucoid by the layer method with solutions of the various antigens in various dilutions. These latter mentioned proteins were prepared according to methods utilized by Hektoen and Cole.[7] Four of the experimental animals produced no precipitins for ox albumin, but precipitins were present for egg white and ovomucin. Three days later the attempt was made to raise the titer by injection intravenously of 30 cc of egg white in three of the animals (#75, 52 and 70) and 50 cc in a 4th (#75) and 35 cc in #22. These latter two animals died at 5- and 6-hour intervals, respectively. When dog #22 was injected intravenously for the third time with 35 cc of egg white solution and died, its liver and spleen were engorged on autopsy and the lungs were pale pink in color. When dog #75 was injected intravenously for the third time with 50 cc of egg white solution, the animal stopped breathing for 45 seconds. Breathing then became labored. The animal became rigid for a few moments and then became relaxed and remained so. Respirations continued to

5. Marshall, J. and Welker, W. H. 1913, J. Am. Chem. Soc. **35**: 820–822.
6. Tracy, G. and Welker, W. H. 1915, J. Biol. Chem. **22**: 55–57.
7. Hektoen, L. and Cole, A. G. 1928, J. Infect. Dis. **42**: 1–24.

TABLE 1.—*Precipitin response in 5 dogs injected intramuscularly with ox albumin adsorbed on aluminum hydroxide cream and 1% egg white solution intravenously (second experiment).*

	Dog	Ox albumin	Egg white	Fowl albumin	Egg albumin	Ovomucin	Ovomucoid
10-29-42	22	2	4	0	0	3	0
	76	0	1	0	0	0	0
	52	4	1	0	0	0	0
	75	3	2	0	0	3	0
	70	1	1	0	0	4	0
11- 5-42	76	0	2	0	0	0	0
	52	0	2	0	0	3	0
	70	0	1	0	0	2	0
11-12-42	76	0	2	0	0	2	0
	52	0	0	0	0	0	0
	70	0	0	0	0	0	0
11-30-42	76	0	0	0	0	0	0
	52	0	0	0	0	0	0
	70	0	1	0	0	0	0

0 = no reaction.
1 = 1:100.
2 = 1:1000.
3 = 1:10,000.
4 = 1:100,000.

TABLE 2.—*Precipitin response in three dogs injected intramuscularly with ox albumin adsorbed on aluminum hydroxide cream and 4 intravenous injections of 1% egg white solution at three-day intervals (third experiment).*

	Dog no.*	Ox albumin	Egg white	Fowl aloumin	Egg albumin	Ovomucin	Ovomucoid
12-23-42	56	0	1	0	1	0	0
	66	0	2	0	0	0	0
	68	2	1	0	0	0	0
12-30-42	56	3	2	0	0	2	0
	66	2	2	0	0	3	0
	68	2	2	0	0	2	1
1- 6-43	56	0	0	0	0	0	0
	66	0	3	0	0	3	0
	68	0	0	0	0	0	0

0 = no reaction.
1 = 1:100.
2 = 1:1,000.
3 = 1:10,000.
* On 1-13-43 there was no reaction between any of the antigens and the serums of dogs #56, 66 and 68.

TABLE 3.—*The appearance and disappearance of serum precipitins.*

Days after last injection of egg albumin solution	Ox albumin					Egg white					Fowl albumin					Egg albumin					Ovomucin					Ovomucoid				
	Dogs					Dogs					Dogs					Dogs					Dogs					Dogs				
	9	75	70	2	22	9	75	70	2	22	9	75	70	2	22	9	75	70	2	22	9	75	70	2	22	9	75	70	2	22
3	0	0	3	0	0	2	2	3	2	0	0	0	0	0	0	0	0	0	0	0	3	3	4	3	0	0	0	0	0	0
4	3	3	2	3	0	2	2	2	2	0	0	0	0	0	0	0	0	0	0	0	3	3	3	3	0	0	0	1	2	1
5	1	0	3	1	0	2	1	2	1	0	0	0	0	0	0	0	0	0	0	0	2	2	2	3	0	0	0	0	1	0
6	0	0	0	2	0	2	1	0	1	0	2	0	0	0	0	0	0	0	0	0	2	2	2	3	0	0	0	0	0	0
7	0	0	4	0	0	3	0	2	3	2	0	3	0	0	0	0	0	0	0	0	2	0	3	2	0	0	0	0	0	0
8	1	0	2	2	0	3	1	4	1	0	0	0	0	0	0	0	0	0	0	0	5	2	4	4	0	0	0	0	0	0
9	2	0	2	2	0	1	1	4	1	0	0	0	2	0	0	0	0	0	0	0	2	0	3	4	0	0	0	0	0	0
10	2	0	2	0	0	1	1	4	2	0	0	0	2	0	0	0	0	0	0	0	2	0	3	3	0	0	0	0	0	0
11	0	0	2	2	3	1	1	1	1	1	0	0	0	0	2	0	0	0	4	0	2	0	3	2	0	0	0	0	0	0
12	0	0	0	2	3	1	1	1	3	2	0	0	0	0	0	2	0	0	2	0	2	0	0	3	0	0	0	0	0	0
13	0	0	0	2	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	3	0	0	2	0	0	0	0	0	0
15	0	0	0	3	0	0	0	0	2	0	0	0	0	4	0	0	0	0	4	0	0	0	0	2	0	0	0	0	2	0
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0

0 = no reaction.
1 = 1:100.
2 = 1:1,000.
3 = 1:10,000.
4 = 1:100,000.

Each of the dogs received intramuscular injections of 30 cc ox albumin adsorbed on aluminum cream and intravenous injections of 1 cc per kg of 1% egg white solution. They received subsequently three additional intravenous injections of egg white solution of the same dosage at three-day intervals.

be labored and the animal died within 5 hours. Autopsy showed enlarged, engorged liver and spleen. The lungs were pale white in color and avascular. No precipitins were present in the animals that remained alive. The results of this experiment are shown in detail in table 1.

In the third experiment three dogs (#56, 66 and 68) were injected intramuscularly with 50 cc of ox albumin adsorbed on aluminum hydroxide cream and were given 4 doses intravenously of 1% egg white solution (1 cc per kg of weight, which was 11.4 cc per animal) at three-day intervals. Six days after the last injection the blood serums were tested for precipitins for ox albumin, and only one of the animals (#68) showed the presence of this precipitin, but all the dogs showed precipitins for egg white. One week later all experimental animals had precipitins for ox albumin, egg white and ovomucin. The precipitin for ox albumin had disappeared the following week and did not reappear the next week. The precipitins against egg white are directed mainly against ovomucin. The results are shown in table 2.

In our next experiment the precipitin titer of the serums was studied in an attempt to obtain more detailed information regarding its appearance and disappearance. Five dogs were injected intramuscularly with 30 cc of ox albumin adsorbed on aluminum cream. They also received intravenously a dose of 1 cc per kg of body weight of a 1% solution of egg white. This dose was repeated three times at three-day intervals. Table 3 shows the precipitin titers of the serums of these 5 dogs from the third day of the last injection of egg white solution to the 17th day. We do not understand the irregular appearance and disappearance of the precipitins for ox albumin.

CONCLUSIONS

The results of these experiments show that it is possible to call forth precipitins in blood serum of the dog, when the antigen is administered intramuscularly by the aluminum cream method and at the same time a condition of toxemia is induced by means of intravenous injection of egg white solution.

TOXEMIA AS A STIMULATION FACTOR IN THE PRODUCTION OF ANTIBODIES.

STUDIES ON PRECIPITIN PRODUCTION IN THE DOG.

II. THE USE OF RICIN

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In a previous communication[1] we described the use of egg white as an aid in precipitin formation in the dog. In order to further investigate the role of toxemia in the formation of precipitins, ricin was selected as the toxin. Since there was none of this protein on the market it was prepared according to the method of Osborne et al[2] from 10 pounds of castor beans, with the only modification that dialysis was carried out against frequent changes of distilled water instead of against running water.

To determine the toxicity of our preparation we used white rats of the Sprague-Dawley strain and found that 0.02 and 0.05 mg per kg of ricin dissolved in physiological saline solution injected subcutaneously was fatal. However, these rats did not die with a subcutaneous dose of 0.001 mg per kg but showed symptoms of toxicity for a period of about 5 days. On the other hand, we found that a dose of 0.001 mg per kg dissolved in physiological saline solution and administered subcutaneously to dogs was fatal in a period of 2 to 4 days. However, a single injection of a dose of 0.5 μg (0.0005 mg) per kg did not kill the dogs but produced symptoms of toxicity over periods of 8 to 14 days, which we found to be a sufficient length of time for the formation of antibodies. Two of our dogs died three days after injection with a dose of 0.0005 mg per kg.

EXPERIMENTAL

In our first experiment 8 dogs were injected intramuscularly with 30 cc of ox albumin fixed on aluminum cream according to the method of Hektoen and Welker[3] described in a previous communication,[1] and simultaneously a single injection of 0.0005 mg per kg of ricin dissolved in physiological saline was administered subcutaneously. Blood was drawn 9, 14, 17, 21, 24, 26, 30, 35, 42, 45, 47 and 49 days after injection. In one animal (#2835, weight 20 lb.) precipitins were found to be present in the blood serum for ox albumin 9 days after injection with a titer as high as 1:100,000. Fourteen days after injection the titer dropped to 1:10,000 but 17 days after injection returned to 1:100,000 once again, whereas 21 days after injection the titer was 1:10,000. The precipitins had disappeared 24 days after injection but had persisted for from 9 to 21 days after injection (more than 12 days). The precipitins were run by the layer method with the antigen in various dilutions.

Another animal (#2836, weight 20 lb.) had precipitins appearing in its blood serum 14 and 17 days after injection in titers of 1:1,000 and 1:100,000, respectively, but none were detected 21 days after injection. These were present for more than three days.

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1. Mann, L. S. and Welker, W. H. 1949, J. Infect. Dis. **85**: 146–149.
2. Osborne, T. B., Mendel, L. B. and Harris, I. F. 1905, Am. J. Physiol. **14**: 259–286.
3. Hektoen, L. and Welker, W. H. 1933, J. Infect. Dis. **53**: 309–311.

A third animal in this series (#2834, weight 30 lb.) showed precipitins 14 days after injection in a titer of 1:10,000, where it remained at that level for several subsequent bleedings. This animal had precipitins for ox albumin in its blood stream for more than 45 days after injection. The blood serum on the 47th and 49th day after injection contained no precipitins for ox albumin.

One dog (#2940, weight 17 lb.) had precipitins for ox albumin in its blood stream 9 days after injection in a titer of 1:100. No precipitins for ox albumin could be detected in subsequent bleedings. Another dog (#3157, weight 21 lb.) did not have precipitins for ox albumin present in its blood stream 9 days after injection, but precipitins for this protein were detected 14 and 17 days after injection in a titer of 1:10,000. Subsequent bleedings showed no precipitins for ox albumin. Dog #3380 (weight 18 lb.) had precipitins for ox albumin in titers of 1:100 and 1:10,000 on the 9th and 14th days after injection, respectively, but none were detected on the 17th day after injection or thereafter. Dog #3404 (weight 20 lb.) had no precipitins for ox albumin in its blood stream on the 9th day after injection, but on the 14th day had a titer of 1:100,000, which dropped to 1:1,000 on the 17th day and remained at that level until these precipitins had disappeared on the 26th day. Precipitins for ox albumin were detected only on the 14th day after injection in dog #3643 (weight 18 lb.).

In this series of experiments precipitins for the material fixed on aluminum hydroxide cream appeared from 9 to 14 days after injection. These persisted from more than 3 days to more than 45 days. The results obtained in the three dogs giving the best responses are expressed in table 1.

After the precipitins had disappeared from the blood stream of the dogs, we attempted to make these animals toxic once again in order to determine if we could recall the antibodies for ox albumin. Carmichael[4] has shown immunity to ricin is produced in rabbits. We found that these dogs had built up a very great resistance or immunity to ricin. Each subsequent injection seemed to confer greater immunity. When an animal showed symptoms of toxicity again, the degree of toxicity was very much less severe and of much shorter duration than originally observed with the subcutaneous injection of 0.0005 mg per kg (0.5 μg). No precipitins for ox albumin could be detected in the blood stream of these animals during this period.

TABLE 1.—*Appearance and disappearance of precipitins for ox albumin in dogs injected intramuscularly with oxalbumin fixed on aluminum hydroxide cream and 0.0005 mg per kg of ricin subcutaneously.*

Days after injection*	Dog #2835	Dog #2834	Dog #3404
9	4	0	0
14	3	3	4
17	4	4	2
21	3	3	2
24	0	3	2
26	0	3	0
30	0	3	0
35	0	3	0
42	0	3	0
45	0	3	0

0 = no reaction.
1 = 1:100.
2 = 1:1000.
3 = 1:10,000.
4 = 1:100,000.
* There was no reaction in any of the dogs after 45 days following injection.

In one animal (#2835) doses of ricin up to 0.275 mg per kg had no effect, whereas this dose, which was given 43 days after the original injection, only made the animal slightly toxic for a period of 24 hours. No precipitins for ox albumin appeared in its blood stream during the following two weeks. Subse-

4. Carmichael, E. B. 1929, J. Pharmacol. **35**: 223–339.

quent injections of ricin had no effect on this animal until a dose of 2 mg per kg was reached, and then the animal was only slightly toxic for a period of 4 days. This injection was given 59 days after the original one was administered. A dose of 4 mg per kg given 65 days after the original injection made the animal slightly toxic for a period of 3 days, but no antibodies to ox albumin were detected in the animal. A subsequent injection of 15 and 25 mg per kg, respectively, had no toxic effect.

TABLE 2.—*Immunity to ricin in one of previously injected animals.*

Dog. #2836	
Days after original injection	Dose in mg per kg
29	0.0005
31	0.001
36	0.100
39	0.125
42	0.175
43*	0.250
56	0.450
57	0.700
58	0.750
59	1.500
60	2.000
63	3.500
64	5.000
65	6.500
66	9.000
67	10.000
70	15.000
72	25.000

* Slightly toxic for 24-hour period following this injection.

Another animal, #2836, showed slight toxicity for a period of 24 hours following an injection of 0.250 mg per kg given 43 days after the initial injection. It had no precipitins present in its blood stream during the two-week period following this injection. Subsequent doses up to and including 25.00 mg per kg had no effect.

Dog #2940 showed slight toxicity for 24 hours following an injection of 0.500 mg per kg 33 days after the original injection. A dose of 2.00 mg per kg made this animal slightly toxic for a 4-day period. No precipitins appeared in its blood stream two weeks following this injection. This injection was given 37 days after the original injection. Subsequent doses of 4, 8, 10, 15 and 25 mg per kg had no effect.

Dog #2834 was slightly toxic for a period of three days with a dose of 2.00 mg per kg (given 60 days after the original injection). Subsequent injections up to and including 15 mg per kg showed no effect. Twenty-five mg per kg given 72 days after the original injection made the animal toxic for three days, and then it died. Dog #3157 was injected with 0.300 mg per kg of ricin 21 days after the original injection. It was toxic for three days and then died. The results with one representative animal (#2836) are expressed in table 2.

DISCUSSION

One theory of action of toxin is that it forms a compound with the antigen, making a foreign protein.[5] Therefore the rabbit reacts to the homologous protein. It is a question as to whether the toxicity produced by the toxin does not predispose the animal towards antibody formation. In our experiments the quantity of toxin used to produce toxicity is so minute that one can hardly conceive of making an effective amount of compound with the antigen. Large quantities of the toxin were used after immunity had been established. No further precipitin was produced although the antigen was still circulating in the blood stream of the experimental animal. It seems highly probable that the toxic state of the animal and not the toxin itself is the basis for the production of the precipitin antibody, since in the absence of the toxic condition of the animal the ricin does not stimulate the formation of precipitin antibody.

We do not know the nature of the

5. Burky, E. L. 1934, J. Allergy, 5: 466–475.

antibody to ricin. Wells[6] thinks this is a neutralizing antibody. Since we have found normal dog serum to contain precipitating factors for ricin, we cannot say whether or not precipitins are formed for this protein.

CONCLUSIONS

1. Precipitins for ox albumin can be produced in the dog following an intramuscular injection of ox albumin fixed on aluminum cream, concomitant with a single subcutaneous injection of 0.0005 mg per kg of ricin.

2. The toxic state appears to be the potent factor in the production of precipitin antibodies in the dog rather than the toxin itself.

3. Dogs develop an immunity to ricin.

6. Wells, H. G. 1925, The Chemical Aspects of Immunity, New York, Chemical Catalog Co.

PHYSIOLOGICAL FACTORS ASSOCIATED WITH GENETIC RESISTANCE TO FOWL TYPHOID

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Some individuals within a species are relatively immune to an infectious disease, while other individuals succumb rapidly when exposed. This observation has induced a number of investigators to study the genetic nature of disease resistance. Their success in establishing genetically resistant and susceptible strains within a number of animal species has recently been extensively reviewed by Gowen (1948). The inheritance of disease resistance with few exceptions is comparable with that for other quantitative characters. Many genes differentiate strains possessing different levels of resistance.

Inbred strains within a species differentiated genetically in their resistances to a disease provide excellent material for investigating the physiological bases of disease resistance. Experiments may be designed to search out the inherited physical characteristics that determine resistance. The defense mechanisms possessed by the recipients of resistant genes may be contrasted with their absence in individuals of susceptible strains of the same species. The facts so obtained could be of major importance in disease prevention. They would also contribute to a better understanding of the physiological nature of gene action.

Metchnikoff's extensive studies on natural and acquired immunity as summarized in 1907 assigned a major role to microphages or polymorphonuclear leucocytes. Temperature effects on the fowl's immunity to anthrax were demonstrated by Pasteur et al as early as 1878. These effects were linked with phagocytic activity by Wagner's observation (1890) that the induced susceptibility was due to a reduction in the leucocytes' phagocytic activity at the low temperature. Ledingham (1908) observed increasing phagocytosis with elevations in temperature from 18 to 42 C. In guinea pig and rabbit polymorphonuclear leucocytes, Harmon et al (1946) observed in vitro enhanced phagocytosis of *Staphylococcus aureus* with each increment of 5 over a temperature range of 22 to 42 C. At 43 C and beyond, phagocytosis rapidly declined.

But ingestion of bacteria by phagocytes is only a step in the control of the invading organisms. If phagocytes are unable to digest the bacteria, they will transport them to various parts of the body where, upon death of the phagocytes, liberated bacteria will initiate new foci of infection as postulated by Werigo (1894) and Bordet et al (1897). In fact, the phagocytes may protect the ingested bacteria, as recognized by Rous and Jones (1916).

Rapid intracellular digestion of *Bacterium typhosum* by rabbit leucocytes

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This report is one of a series of investigations on genetic resistance to fowl typhoid that are being carried on in the Genetics Laboratory at the Iowa State College under the direction of Dr. John W. Gowen.

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was observed in vitro and reported by Mudd et al (1933). Within 15 minutes after ingestion, many of the phagocytosed bacteria were swollen, stained faintly or irregularly, and often appeared as mere indistinct shadows which could scarcely be recognized. Cottingham and Mills (1943) in vitamin deficiency studies on mice, white rats, and guinea pigs observed that after one-hour digestion in vitro, a majority of the polymorphonuclear leucocytes containing bacteria showed evidence of intracellular lysis. Type I pneumococcus, a coagulase-positive staphylococcus, and *Micrococcus candidus* were used in their experiments.

In view of the above studies, variation in body temperature or digestive ability of phagocytes could influence host resistance. The present study was designed to investigate the possible dependence of resistance of the fowl to *Salmonella gallinarum*, the etiological agent of fowl typhoid, on genetically controlled differences in body temperature and phagocytic activity of polymorphonuclear leucocytes.

MATERIALS AND METHODS

The resistant strains of chicks used were those developed and maintained by the Genetics Department at Iowa State College (Lambert and Knox, 1928 and 1932; Lambert, 1932; Gowen, 1937–47). Close inbreeding over a number of years has firmly fixed the degree of genetic resistance. Three of the resistant strains (A, B, and C) were of the White Leghorn breed, while the fourth resistant strain, RR, was developed from a White Leghorn-Rhode Island Red cross followed by selective breeding for resistance. The susceptible strain (S) was of the New Hampshire breed and was obtained either as eggs or one-day-old chicks. Comparative reactions of the strains to virulent *S. gallinarum* are shown in table 1.

Chicks were inoculated intraperitoneally at 10 days of age, and deaths were recorded thereafter for 21 days. Highest mortality occurred from 5 to 7 days after inoculation. Relatively few deaths were observed the last week of the test period. Table 1 reveals a uniformly high resistance for the 4 resistant strains. The susceptible chicks are equally uniform in their reaction to a much smaller inoculum, but in the susceptible direction. In studies reported here resistant chicks were from strain A, unless otherwise designated.

The cultures of *S. gallinarum* were kept in a high state of virulence by frequent reisolation from chicks dying following inoculation. Cultures consisting of gram-negative short rods that produced acid but no gas in dextrose and maltose and neither acid nor gas in sucrose and lactose were considered to be *S. gallinarum*. Generic identification was also made with appropriate antiserum.

Bacterial suspensions for inoculations, as well as for other studies, were obtained from 18-hour growth on veal infusion slants. Bacteria were suspended in physiological salt solution containing sufficient bactopeptone (0.05%) to maintain the number of viable bacteria. Concentration of bacteria was estimated with a Gates nephelometer and appropriate dilutions were made. Actual numbers of viable organisms were determined by poured agar dilution plates.

TABLE 1.—*Comparative resistance of genetically differentiated strains of chicks to* S. gallinarum.

Strain	Inoculated dose	Total	Survived	Percent survival
A	2×10^8	745	636	85
B	2×10^8	181	172	95
C	2×10^8	221	194	88
RR	2×10^8	167	145	87
S	2×10^2	657	100	15

All differences between resistant strains and S are highly significant ($P<0.01$).

Chicks were reared in battery brooders in which the temperature was maintained by thermostatic control at about 30 C. A standard chick mash was fed all chicks.

Temperatures of chicks were determined rectally by means of a specially constructed thermo-couple and were recorded to the nearest 0.1 C. Unless otherwise shown, temperatures were taken between 9:00 and 12:00 a.m. daily. All temperatures given in this report are for the centigrade scale.

Polymorphonuclear leucocytes for in vitro phagocytosis and intracellular digestion studies were obtained from normal chicks approximately 8 weeks of age. A sterile leucocytic exudate was induced in the peritoneal cavity by injection of 25 ml of a starch aleuronat paste mixed with an equal volume of tryptose broth. After 18 hours chicks were exsanguinated by bleeding from the heart, and the serum recovered was used in the

phagocytic system. The peritoneal exudate was suspended in physiological salt solution containing heparin diluted 1:500. The suspension was then centrifuged gently and standardized by means of the Neubauer counting chamber to about 50,000 cells per cmm. Leucocytes were used within three hours after recovering from chicks. A fresh bacterial suspension was made, as described above, for each experiment and was diluted to an estimated 5,000,000 bacteria per ml. Leucocytes and serum were first pipetted into paraffined tubes (73 by 8 mm), and bacteria were added just before the phagocytic test was made. One-tenth ml of each component was added and the tubes were sealed with paraffined corks. The tubes were immediately placed in a water bath and agitated with a lateral motion (120 reversals of direction per minute) for 10 minutes. A sample was then removed and smeared on a slide. For intracellular digestion studies, immediately after sampling a tube it was placed again in the water bath and left undisturbed for one hour in order that digestion of phagocytosed bacteria might take place. A second smear was then made as before. All phagocytosis and digestion studies were made at 41 C, except in experiments involving temperature effects. In these experiments additional tubes were run at 36 and 46 C. Chicks from resistant and susceptible strains were equally represented in each experiment.

After drying, the slides were stained with Pappenheim's pyronine-methyl green stain. On each smear 4 samples of 100 polymorphonuclear leucocytes were examined for the presence of phagocytosis. The method of evaluating the phagocytic reaction was that of Hamburger (1912), which involved the determination of percent leucocytes containing bacteria. Digestion slides were counted in the same manner; any ingested bacterium that took enough stain to be recognizable was considered as not digested. An estimate of the digestive ability for each individual was obtained from the ratio of number of leucocytes containing bacteria before digestion to the number of leucocytes containing bacteria after digestion. The relative efficiency of this method as compared with other possible methods will be discussed later.

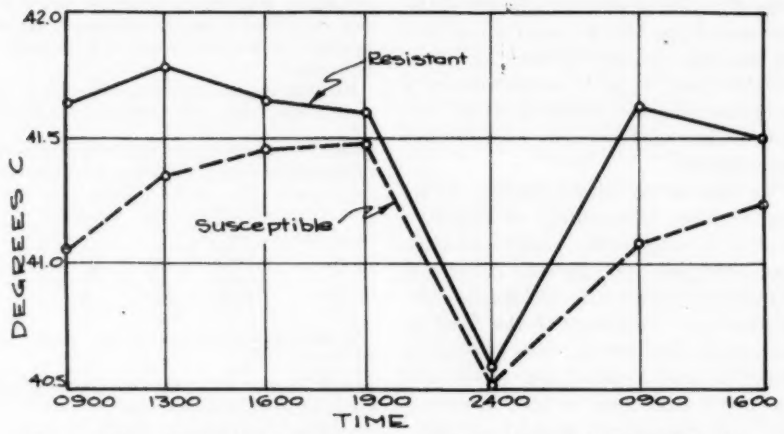

FIG. 1.—Diurnal fluctuations in normal temperature of resistant and susceptible strains.

BODY TEMPERATURE AND GENETIC RESISTANCE

In a preliminary study no significant differences were observed in normal body temperatures of resistant and susceptible strains (Bell, 1947). Temperatures were recorded between 7:00 and 9:00 p.m. In view of the rather large diurnal variation reported for the fowl (Fronda, 1921; Lamoreux and Hutt, 1939), more extensive experimentation appeared desirable.

Diurnal fluctuations in normal body temperature.—In order to establish the diurnal variation in temperature for both resistant and susceptible strains,

body temperatures of 12 resistant and 6 susceptible 10-day-old chicks were taken at various times over a period of two days. Figure 1 and table 2 reveal significantly higher temperatures for resistant chicks than for susceptible individuals; however, this difference was evidenced only during daylight hours. At night when metabolism was low due to inactivity, the temperatures of all chicks were reduced to a common level of about 40.5 C. Greatest difference between strains occurred during the morning. Individuals of the resistant strain reached their peak temperature by noon, while susceptible chicks showed a steady increase in temperature until late afternoon. This experiment indicated that for strain comparisons data should be taken within a relatively narrow time range, preferably between 9:00 a.m. and noon.

TABLE 2.—*Diurnal fluctuations in normal body temperature of 10-day-old resistant and susceptible chicks.*

Time of day	Mean body temperature (degrees C)		Difference
	Resistant	Susceptible	
0900	41.62±0.15	41.05±0.06	+0.57*
1300	41.79±0.12	41.35±0.09	+0.44*
1600	41.66±0.10	41.45±0.16	+0.21
1900	41.62±0.14	41.48±0.21	+0.14
2400	40.59±0.10	40.50±0.23	+0.09
0900	41.64±0.13	41.08±0.07	+0.56†
1600	41.53±0.08	41.25±0.10	+0.28*
Average mean difference =0.33 +0.08†			

* Significant at 5% level.
† Significant at 1% level.

Variation in normal body temperature over test period.—Daily body temperatures were recorded from 9 to 23 days of age for 15 chicks of strains A and RR, 10 chicks from C, and 13 from the susceptible strain, S. Observations were made daily between 9:30 and 11:00 a.m. thus excluding diurnal variation. Mean daily temperatures for each strain are shown graphically in figure 2. In addition to differences between strains, significant variation was present within strains between days. The relative importance of these sources of variation can be recognized from the analysis of variance shown in table 3. The highly significant mean square due to strains can be attributed to the fact that, in addition to susceptible chicks having a low temperature, resistant strains differed from each other. The breeding program practiced had fixed in each strain its own particular mechanism for regulating body temperature. The average temperatures varied from 41.5 for strain C to 41.0 C for the susceptible strain, S. Strains RR and A had intermediate temperatures with values of 41.3 and 41.2 C, respectively.

TABLE 3.—*Analysis of body temperature for period of 9 to 23 days.*

Source variation	Degrees of freedom	Mean square	Components of mean square
Total	794		
Strains	3	5.65†	E+13.2I+197.2 S
Days	14	0.94†	E+13.2I+53 D
Strains×days	42	0.11	E+13.2I
Within	735	0.09	E

Source of variation	Percent of total variance
Strain effect, S	21
Day effect, D	12
Strain by day interaction, I	1
Independent, E	66

† Significant at 1% level.

The large variation attributed to days was in part due to a slight increase in body temperature with age, but it is apparent from figure 2 that the age contribution was small. The major cause of variation between days was inherent in

the experimental setup. Chicks were reared in brooders with temperature thermostatically controlled, but the chicks had access to an unheated portion from which feed and water were obtained. They occupied this section during most of the daylight hours, and daily body temperatures were associated with variations in room temperature. Fortunately, strains reacted in like manner to

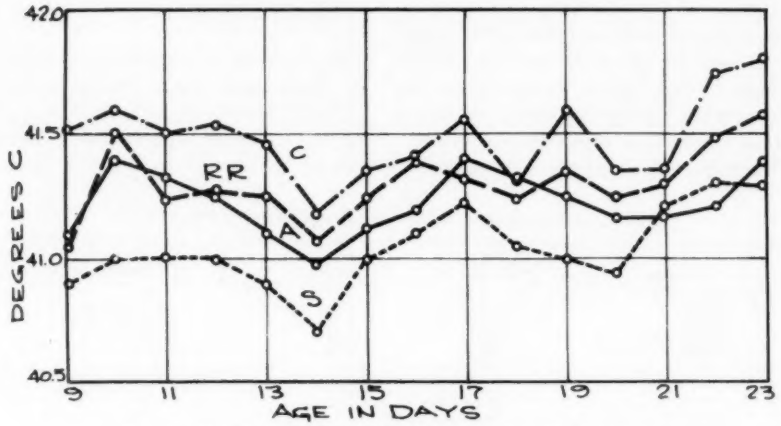

FIG. 2.—Normal temperatures of strains from 9 to 23 days of age.

environmental temperature fluctuations as indicated by the small strain by day interaction. Differences between strains, the primary interest here, were not confounded by the daily variation. When total variance for body temperature was separated into its component parts, it was found that independent causes within strains accounted for 66% of the total, strain differences 21%, day differences 12%, and strain by day interaction only 1%. Genetic control of 21% compares favorably with other physiological characteristics. Gowen and Calhoun (1943) reported strain differences in mice accounted for 26% of variance in total leucocytes and 22% in erythrocytes.

The relative resistance of various strains (table 1) can now be compared with their normal body temperatures as evidenced in the above experiment. Resistant strains revealed only slight differences in mortality, but these differences, even though they are small, closely paralleled differences in body temperatures. Susceptible chicks received an inoculum many times smaller than the resistant strains. The association of susceptibility and low body temperature is obvious. These data reveal a positive correlation of 0.83 between body temperature and fowl typhoid resistance, but as this correlation is based on but few degrees of freedom, it should not be overstressed.

Body temperature during the course of fowl typhoid.—The association of high normal body temperature with genetic resistance to fowl typhoid concurs with reported studies on genetic resistance to *Salmonella pullorum* (Scholes and Hutt, 1942; Severens et al, 1944). Since resistance is determined by a differential response of strains to the disease entity, it appeared desirable to study temperature response following inoculation. Twenty-one resistant and 8 susceptible

chicks were inoculated at 10 days of age. Daily temperatures were recorded from 9 to 24 days. In order to detect deviations from normal, 19 resistant and 8 susceptible control chicks were included in the experiment. Results are shown in table 4 and figure 3. Higher normal temperatures in resistant chicks, as shown in control comparisons, were again evidenced. Most of the daily but in the later disease stages the relationship was negative.

Within a strain, chicks that survive can be considered more resistant than those dying. Of the inoculated susceptible chicks in one experiment, 6 survived and 9 died. Daily mean temperatures for these two groups are illustrated in figure 4. The trend is similar to that observed between resistant and suscepti-

TABLE 4.—*Body temperatures of resistant and susceptible strains (controls and inoculated).*

Age of chicken (days)	Controls			Inoculated		
	Mean body temperature (degrees C)			Mean body temperature (degrees C)		
	Resistant	Susceptible	Difference	Resistant	Susceptible	Difference
9	41.32±0.05	41.22±0.06	+0.10	41.33±0.04	41.26±0.04	+0.07
10	41.25±0.05	40.99±0.07	+0.26†	41.27±0.07	41.02±0.07	+0.25*
				Inoculated		
11	41.19±0.04	41.00±0.08	+0.19*	41.50±0.08	41.08±0.06	+0.42†
12	41.11±0.05	40.86±0.04	+0.25†	41.58±0.08	41.14±0.13	+0.44†
13	41.34±0.04	41.25±0.06	+0.09	41.63±0.05	41.22±0.23	+0.41*
14	41.46±0.10	41.56±0.11	−0.10	41.58±0.07	41.60±0.14	−0.02
15	41.19±0.05	41.18±0.09	+0.01	41.45±0.05	41.74±0.09	−0.29*
16	41.33±0.07	41.16±0.16	+0.17	41.42±0.04	41.72±0.24	−0.30*
17	41.27±0.06	41.10±0.06	+0.17	41.44±0.06	42.05±0.11	−0.61†
18	41.36±0.04	41.08±0.06	+0.28†	41.52±0.06	41.95±0.12	−0.43
19	41.48±0.05	41.24±0.08	+0.24*	41.68±0.05	42.20	−0.52
20	41.37±0.04	41.10±0.06	+0.27†	41.44±0.04	41.90	−0.46
21	41.39±0.05	41.16±0.05	+0.23†	41.42±0.04	41.50	−0.08
22	41.25±0.03	41.02±0.07	+0.23†	41.52±0.06		
23	41.41±0.04	41.18±0.07	+0.23†	41.44±0.11		
24	41.35±0.05	41.11±0.06	+0.24†	41.47±0.04		
	Average mean difference =0.18±.03†					

* Significant at 5% level.
† Significant at 1% level.

mean differences between the controls were statistically significant ($P = <0.05$), and the average daily mean difference of 0.18 C was highly significant ($P = <0.01$). Results from inoculated chicks were more surprising. For three days following inoculation resistant chicks maintained significantly higher temperatures, but by the 5th day this relationship was reversed, with susceptible chicks exhibiting a much higher temperature until all had died by the 11th day. Within the resistant strain 90% survived the test.

The above experiment was repeated with similar results. Resistant chicks invariably possessed higher temperatures during the disease incubation period, ble strains (figure 3). Survivors had higher temperatures immediately following inoculation, but those subsequently dying developed significantly higher temperatures on the 4th, 5th, and 6th days. No significant differences were evidenced between the two groups before inoculation. Within the resistant strain similar results were observed. Immediately after inoculation a rapid differentiation occurred. Those destined to survive maintained higher temperatures for the first three days. Thereafter, chicks subsequently dying consistently possessed the higher body temperature.

Effect of artificially reduced body temperature on resistance.—Another method

of testing the correlation between genetic resistance and body temperature is offered when body temperature is reduced by lowering brooder temperature of chicks brooded at 24 C. Both resistant and susceptible chicks reared at the lower temperature possessed significantly lower body temperatures than

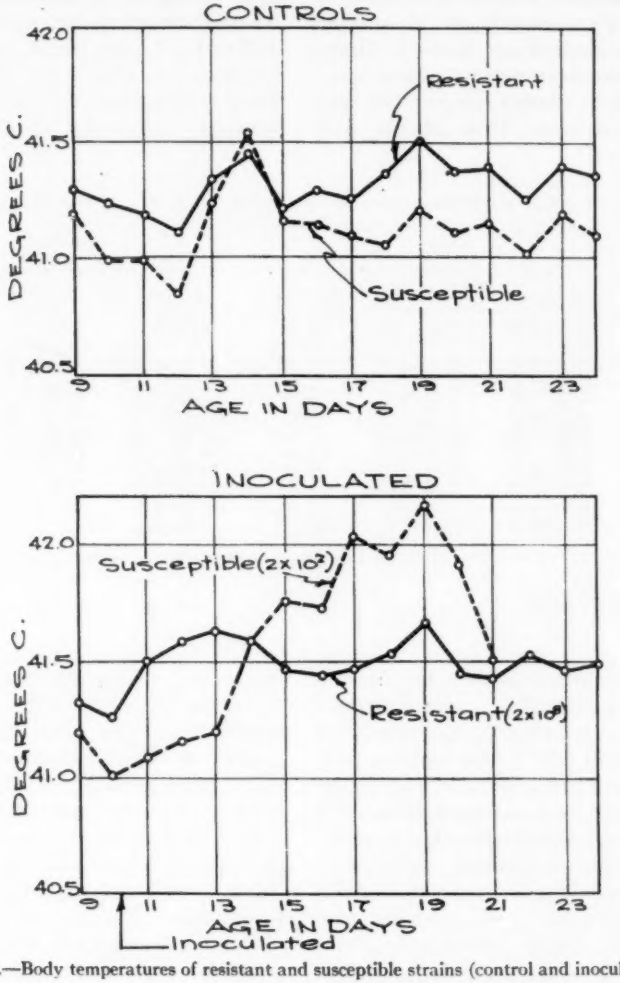

FIG. 3.—Body temperatures of resistant and susceptible strains (control and inoculated).

ture. Chicks of resistant and susceptible strains were brooded at 30 and 24 C following inoculation. Body temperatures were recorded daily for 11 days. Results presented in table 5 and figure 5 indicate a significant decrease in body temperature their corresponding controls. The average difference between resistant groups was 0.24 as compared to 0.47 C between susceptible groups. Thus the susceptible strain had nearly twice the internal heat loss of their resistant as-

sociates at the low environmental temperature. This indicates a more efficient temperature-regulating mechanism in the resistant strain.

Mortality data (table 6) reveal the effect of hypothermia on resistance to fowl typhoid. Within both resistant and susceptible strains, chicks brooded at the lower temperature were more susceptible. The third experiment was not well adapted for the test desired, as the mortality was so severe that only one chick survived at either temperature. With this exception, mortality was less in every case at the higher temperature. Differences within individual experiments were not statistically significant; however, similar trends were evidenced throughout. Heterogeneity of variances in the mortality data prevented combining experiments for a more adequate test of significance. Among chicks dying, days to death gives another measure of relative resistance. Within the 4 experiments testing the effect of artifically reduced body temperature, all significant differences in mean days to death were in favor of chicks reared at 30 C.

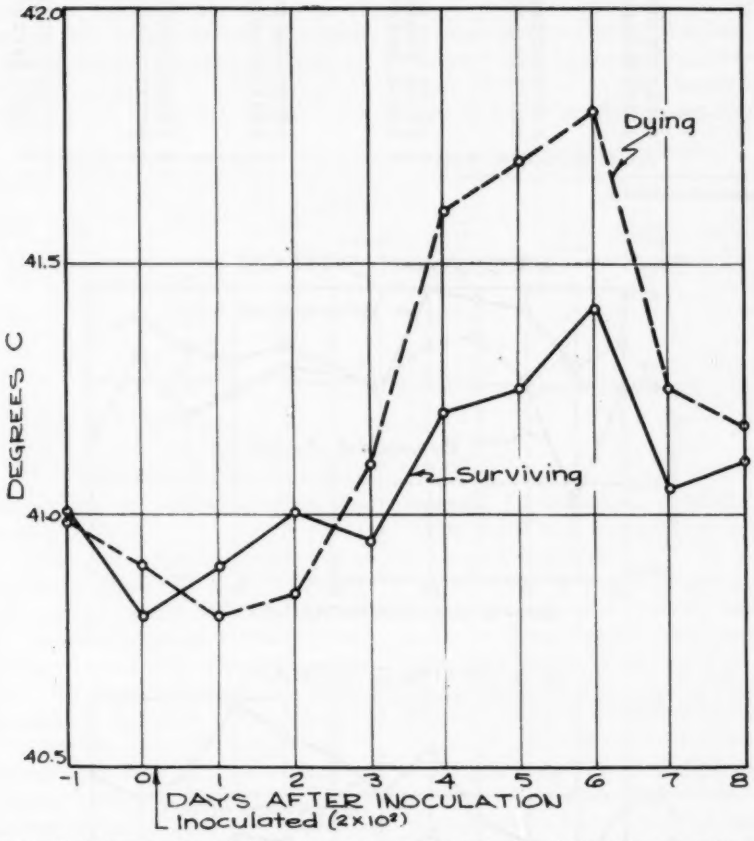

FIG 4.—Body temperatures of chicks that subsequently survived or died within susceptible strain

TABLE 5.—*Temperatures of inoculated resistant and susceptibe strains brooded at 30 and 24 C.*

Days after inoculation	Resistant strain			Susceptible strain		
	Mean body temperature (degrees C)			Mean body temperature (degrees C)		
	Brooded at 30 C	Brooded at 24 C	Difference	Brooded at 30 C	Brooded at 24 C	Difference
0	41.72	41.48	+0.24	41.45	41.13	+0.32
1	41.58	40.92	+0.66	41.11	40.63	+0.47
2	41.90	41.52	+0.38	41.25	40.90	+0.35
3	41.95	41.75	+0.20	41.38	41.04	+0.34
4	42.02	41.72	+0.30	41.75	41.32	+0.43
5	41.45	41.50	−0.04	41.52	41.16	+0.36
6	41.60	41.60	0	41.72	41.14	+0.58
7	41.68	41.60	+0.08	41.79	41.25	+0.54
8	41.58	41.43	+0.15	41.97	41.30	+0.67
9	41.65	41.35	+0.30	41.60	41.10	+0.50
10	41.78	41.65	+0.13	41.48	41.13	+0.35
11	41.70	41.20	+0.50	41.58	40.80	+0.78
	Average mean difference = 0.24 ± 0.06†			Average mean difference = 0.47 ± 0.04†		

† Significant at 1% level.

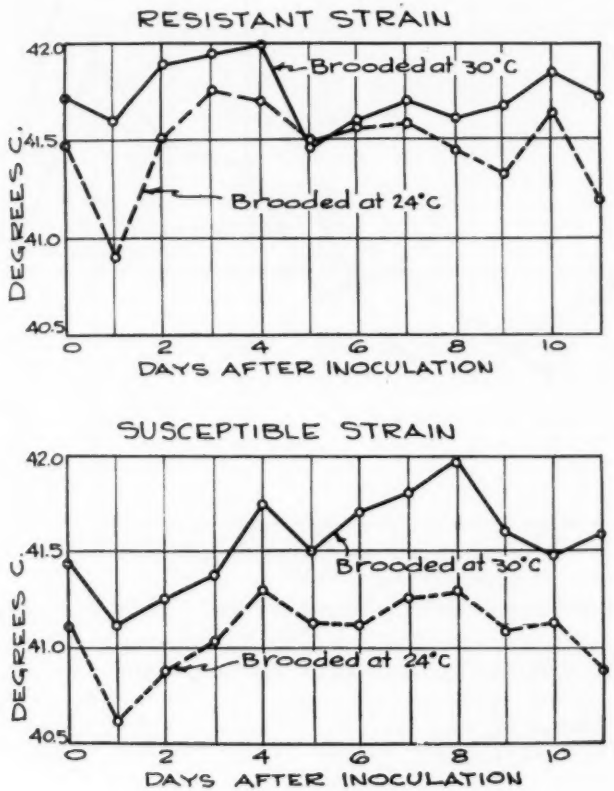

FIG. 5.—Influence of environmental temperature on body temperature in resistant and susceptible strains.

Thus resistance as measured by percent survival and by days to death is lowered by a reduction in body temperature. Differences in mortality of control chicks brooded at the two environmental temperatures were insignificant. These facts indicate that body temperature can have a real effect on resistance to fowl typhoid. The data are concordant with those of Scholes and Hutt (1942) on the effect of reduced body temperature on resistance to *S. pullorum*.

If these data appear contradictory to the results for inoculated chicks in table 4, it should be recalled that the susceptible strain did not possess the higher temperature until 3 to 4 days after inoculation. By then infection has spread throughout the body, and the fever is probably the result of toxic damage to the temperature-regulating mechanism. A fever temperature during this period appears to be ineffective in combating the multitude of pathogens present in the body.

TABLE 6.—*Comparative resistance of inoculated chicks brooded at different temperatures.*

Experiment	Strain	Inoculated dose	Brooded at 30 C			Brooded at 24 C		
			Number of chicks	Percent survival	Mean days to death	Number of Chicks	Percent survival	Mean days to death
I	S	1×10^2	11	46	8.3±0.8	13	23	5.1±0.4
	R	1×10^8	5	80	4*	5	40	7.3±2.2
II	S	1×10^2	20	20	9.1±1.0	20	10	6.6±0.3
III	S	1×10^9	13	0	7.3±0.6	11	9	5.8±0.2
IV	S	1×10^9†	20	60	8.8±1.6	17	47	10.1±1.9
	R	1×10^8	28	61	6.7±1.2	28	32	4.7±0.3

* Based on only one individual.
† Less virulent culture used here to reduce mortality in S Strain.
Difference in mean days to death, 30–24 C:
Experiment I susceptible and Experiment III—significant at 1% level.
Experiment IV resistant and Experiment II—significant at 5% level.

HUMORAL AND CELLULAR FACTORS IN GENETIC RESISTANCE

High body temperature per se has been shown to be insufficient to account for genetic resistance to fowl typhoid (table 4). If it is to be a factor in genetic resistance, it must exert its influence through other defense mechanisms during the incubation period of the disease. One of the more important defense reactions known to be influenced by body temperature is phagocytosis. Before evaluating the relative phagocytic abilities of polymorphonuclear leucocytes from resistant and susceptible strains, tests were made for normal bactericidins. Strain differences in the bactericidal power of whole blood or serum such as reported by Schnetzler and Hartsell (1940) could be a confounding element in phagocytic studies.

Bactericidal properties of whole blood and serum.—Since leucocytes and serums for phagocytosis were obtained from chicks about 8 weeks of age, tests for bactericidins were made at that age. Blood was taken aseptically from the heart and collected in sterile tubes containing sufficient quantity of heparin to prevent coagulation. Bactericidal tests were conducted as follows: A uniform quantity (0.5 ml) of whole blood and 0.1 ml of a bacterial suspension diluted to an estimated 5000 bacteria per ml were placed in sterile agglutination tubes. The number of viable bacteria in the suspension was checked by poured dilution agar plates. Tubes were incubated at 41 C for two hours. Contents of each tube were then mixed with 10 ml of nutrient agar and poured into

petri dishes. Bacterial colonies were counted after 48 hours incubation. All tests were made in duplicate. Typical results are shown in table 7.

Considerable variation was observed between individuals within any strain. Differences between strains were not significant. Similar results were obtained when serum was used in place of whole blood in the bactericidal test. These results indicated that genetic resistance to fowl typhoid at 8 weeks of age is not dependent upon normal bactericidins in whole blood or serum. These tests confirm those which have been conducted over a period of years by other workers in this laboratory.

TABLE 7.—*Bactericidal action of whole blood of various strains on* S. gallinarum.

Experiment	Number of individuals per strain	Expected colonies per plate	Colonies per plate			
			A	C	RR	S
I	10	500	402	420	534	496
II	10	500	429	383	—	322

For proper interpretation of bactericidal and phagocytic studies the relative resistance of strains at 8 weeks of age was determined. Severens et al (1944) reported that *S. pullorum* resistant and susceptible strains were equally resistant by 10 days of age. Results in table 8 indicate that the differences between strains at 10 days of age in resistance to *S. gallinarum* is still present in 8 week old birds of the same strains.

Phagocytosis by polymorphonuclear leucocytes.—Normal body temperature of the chick is about 41 C. This temperature was chosen for comparative in vitro phagocytosis studies. Adjustment for the difference in body temperatures of resistant and susceptible strains did not seem practical for the in vitro experiments. Leucocytes and serums were obtained from normal individuals, and agglutination tests were made on each serum sample to assure the absence of *S. gallinarum* antibodies. The phagocytic mixture was incubated as previously described. Six experiments were conducted with resistant and susceptible strains equally represented within each experiment. A total of 20 individuals were tested. After the smears were stained, 4 samples of 100 polymorphs from each individual were examined for the presence of ingested bacteria. At the constant temperature of 41 C, resistant and susceptible strains possessed equal phagocytic abilities, with 9.5% and 9.2% phagocytosis, respectively. These values are lower than those usually reported (Mudd et al, 1933; Harmon et al, 1946). They could be characteristic of the fowl, or optimum contact between leucocytes and bacteria may not have been obtained by the method employed. In either case, the evidence indicates no strain difference.

TABLE 8.—*Comparative resistance of strains to* S. gallinarum *at 8 weeks of age.*

Strain	Inoculated dose	Inoculated	Survived	Percent survival
A	2×10^5	27	27	100
B	2×10^5	7	7	100
C	2×10^5	4	4	100
S	2×10^5	17	1	6

Temperature effects on phagocytosis.—Experiments measuring body temperature following inoculation showed that resistant chicks maintained significantly higher body temperatures during the important incubation period of fowl typhoid. Phagocytosis in vivo for resistant chicks must be occurring at a higher temperature than in the susceptible strain. To determine the effect of temperature on phagocytosis, experiments were conducted at temperatures ranging from 36 to 46 C with intervals

of 5 C. Even though the extreme temperatures would not be found in vivo, they provided points from which a trend passing through the normal could be established. With few exceptions polymorphonuclear leucocytes from each chick were tested at all temperatures. Thus, results shown in table 9 are not confounded with differences between individuals.

With each increase in temperature there was a corresponding increase in the phagocytic ability of polymorphs. This increase was evidenced within both strains and appeared uniformly in all individuals.

For tests of significance the percentage data were transformed into angles. This transformation was desirable since the percentage values were small and would not be normally distributed. Corresponding angles should approach a normal distribution. Differences between strains at the various temperatures were not significant, thus confirming the previous results, which had indicated equal phagocytic abilities for resistant and susceptible leucocytes at the uniform temperature of 41 C. Regressions of percent phagocytosis on temperature were highly significant within both strains. A greater number of bacteria were ingested as the temperature of the phagocytic mixture was increased. This suggests greater in vivo phagocytosis in inoculated resistant chicks due to their higher body temperatures. Significantly, this higher body temperature was most prominent immediately following inoculation when the pathogens are relatively few in number.

Intracellular digestion of phagocytosed bacteria.—The above experiments point to greater in vivo phagocytosis in resistant chicks because of their higher body temperature. Enhanced phagocytosis could be detrimental if phagocytes were unable to lyse ingested bacteria. In this case, leucocytes could transport bacteria throughout the body. Upon death of the leucocytes, liberated bacteria would initiate new foci of infection.

TABLE 9.—*Effect of temperature on phagocytosis.*

Chicks by strain	Percent phagocytosis		
	36 C	41 C	46 C
Resistant			
R2378	2.8	8.5	12.5
R2379	—	7.0	16.5
R2390	3.2	5.2	6.5
R2438	3.2	11.5	6.0
R2439	—	6.0	9.8
Average	3.1	7.6	10.0
Susceptible			
S3892	—	10.5	16.5
S3902	—	12.5	19.5
S3903	2.2	4.8	12.5
S9991	2.8	8.5	13.0
Average	2.5	9.1	15.2

The digestive abilities of resistant and susceptible leucocytes were tested in 4 experiments. Resistant and susceptible strains were equally represented. The phagocytic mixture for each individual was sampled as described earlier. All slides were stained for an equal period of time with Pappenheim's pyronine-methyl green stain. Bacteria stained bright red, while nuclear material of the leucocytes appeared as blue. Partially digested bacteria stained lightly and often resembled shadows. Photographs of representative polymorphonuclear leucocytes appear in figures 6, 7, and 8.

Digestive ability for each individual was estimated from the following ratio:

$$\frac{\text{Number of active polymorphs after digestion}}{\text{Number of active polymorphs before digestion}} \times 100 = \text{Digestive ratio}$$

Active polymorphs refer to those containing ingested bacteria. Individuals whose polymorphs lysed a majority of

ingested bacteria would have a small digestive ratio. When no digestion occurred, a value near 100 would be obtained. This crude estimate of digestive ability has some disadvantages which tend to underestimate intracellular digestion. Partially digested bacteria would be considered as not digested. Also, any bacteria phagocytosed during the digestion period would also bias the results toward no digestion. Most methods of estimating intracellular digestion of bacteria involve the differentiation of partially digested bacteria from normal bacteria. The method used in experiments reported here has the advantage of erasing this personal equation. Even though certain errors are involved, the method used should give an unbiased estimate of differences between resistant and susceptible strains.

Individual digestive ratios for resistant and susceptible chicks reveal a remarkable strain difference, as shown in table 10. All individuals within the resistant strain had destroyed more than 50% of phagocytosed bacteria. Susceptible chicks in no case reached the 50% level. Under the conditions of these experiments the strains differed significantly ($P<0.001$) in their lytic capacities.

Some caution should be observed in interpreting in vitro studies. Intracellular digestion here can be taken only as an index to in vivo activity. The less favorable environment outside the body should affect resistant and susceptible leucocytes in a similar manner. Differences between strains as wide as those observed here would be expected to be maintained in the body. This qualitative difference between individual phagocytes of resistant versus susceptible strains is so pronounced that it could largely account for the differences in resistance observed in tables 1 and 8.

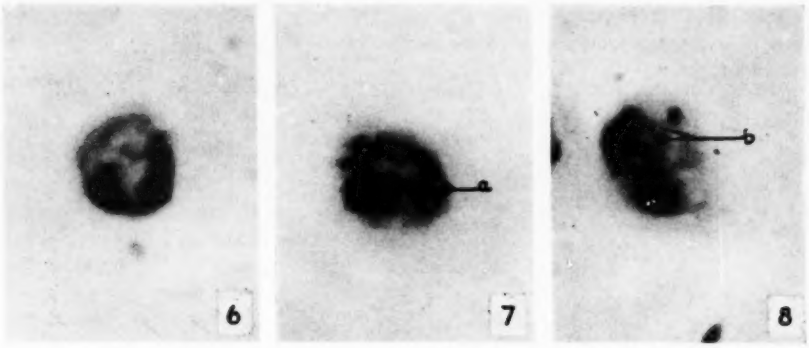

FIG. 6, 7, and 8.—Polymorphonuclear leucocytes showing phagocytosis and intracellular digestion of bacteria. Note normal bacteria (a) in contrast with light-staining and indistinct outline of partially digested bacteria (b). Pappenheim's pyronine-methyl green stain (×3000).

FIG. 6.—Polymorph with no phagocytosis.

FIG. 7.—Polymorph with phagocytosed bacteria.

FIG. 8.—Polymorph showing digestion of phagocytosed bacteria.

DISCUSSION

Genetic resistance to fowl typhoid was closely associated with a higher than average body temperature. Resistant strains consistently evidenced normal temperatures from 0.2 to 0.4 C

higher than susceptible strains. Temperature studies during the disease indicated that high body temperature per se was insufficient to account for genetic resistance (table 4). Susceptible individuals developed much higher fever temperatures during the course of the infection than did resistant chicks. Both between and within strains comparisons showed fever temperatures in the later disease stages to be correlated with susceptibility. However, resistant chicks possessed the higher body temperature at the time of inoculation and during the incubation period of the disease. High body temperature at this time could be a factor in resistance by enhancing the efficiency of such defense mechanisms as phagocytosis while the pathogens are relatively few in number.

Polymorphonuclear leucocytes from resistant and susceptible strains exhibited equal phagocytic abilities at the same temperature, and both strains showed increasing phagocytosis with each elevation of temperature (table 9). Thus the higher body temperature of resistant chicks during the initial disease stages suggests a more rapid rate of phagocytosis than would be occurring in susceptible chicks. Conversely, a greater advantage should be found with susceptible individuals during the late stages of fowl typhoid, but by this time the bacteremia has become so widespread in susceptible individuals that no apparent benefit is derived from the higher temperature.

Additional evidence indicating that body temperature plays a role in disease resistance is provided in experiments artificially reducing body temperature. Resistance is lowered within both resistant and susceptible strains when body temperature is reduced by brooding at low environmental temperatures.

The importance of body temperature in genetic resistance has been considered by other investigators. Scholes and Hutt (1942) concluded it to be a major factor in genetic resistance to *S. pullorum*. Resistant chicks maintained a higher body temperature from 2 to 10 days of age, the period of greatest susceptibility, than did susceptible individuals. Artificially induced hypothermia was followed by a decrease in resistance. Hyperthermia induced by a brooder temperature of 38 C produced a corresponding increase in resistance. Severens et al (1944) also found differences in body temperature between *S. pullorum* resistant and susceptible strains of chicks, but they assigned the major role in resistance to a larger number of lymphocytes in the resistant strain. Their conclusions were substantiated by a number of uniquely designed experiments. These studies are of direct interest here since *S. pullorum* and *S. gallinarum* infections are similar in many respects. Lambert (1930) observed in the fowl typhoid resistant strains used here a greater resistance to *S. pullorum* than was evidenced in control strains. This suggests some common defense mechanisms against the two diseases.

To assign major significance to quantitative factors such as higher body temperature or greater number of lymphocytes assumes that individual units within defense mechanisms of all strains have equal abilities to destroy or lyse localized bacteria. There is little evidence reported to support such an assumption. Studies on the relative intracellular digestion of resistant and susceptible polymorphonuclear leucocytes indicate marked strain differences (table 10). The resistant strain was more than twice as effective as the susceptible strain in destroying ingested bacteria. This qualitative difference in intracellular digestive enzymes could more nearly account for the wide re-

sistance levels to *S. gallinarum* observed in tables 1 and 8. Other phagocytic cells having identical genetic constitution, as the polymorphs, would probably show similar strain differences in intracellular digestion. Such differences have been observed in other animal species. Oakberg (1946) in a study of the livers and spleens of 6 strains of mice ranging from high resistance to almost complete susceptibility to mouse typhoid, *Salmonella typhimurium*, observed increasing ability of macrophages to digest phagocytosed bacteria with increasing genetic resistance.

TABLE 10.—*Intracellular digestion of* S. gallinarum *by polymorphonuclear leucocytes of resistant and susceptible strains.*

Experiment	Individual digestive ratios	
	Resistant strain	Susceptible strain
I	16.7	83.3
	36.8	69.0
	18.2	78.6
II	46.4	95.2
	45.8	74.0
III	47.1	173.7
IV	26.9	58.0
	45.8	69.2
Average	35.5	75.7

If quantitative differences in defense mechanisms were of major importance in specific disease resistance, they should also impart considerable generalized resistance. Except for closely related diseases, generalized resistance does not appear to be widespread (Gowen, 1948). Qualitative differences in intracellular enzymes could give resistance to a specific pathogen without imparting resistance to other pathogens with different chemical compositions.

In genetic resistance to fowl typhoid high body temperature appears to be of secondary importance when compared with the relative digestive abilities of polymorphonuclear leucocytes. Further studies should include lymphocytes as well as wandering and fixed macrophages. These cells could assume equal or greater importance in fowl typhoid resistance.

SUMMARY AND CONCLUSIONS

The relationships of body temperature and phagocytic activity of polymorphonuclear leucocytes to genetic resistance were studied in strains of chicks differentiated in their resistance to *Salmonella gallinarum*, the causative organism of fowl typhoid.

Resistant strains of chicks possessed higher normal body temperatures during the test period, 9 to 23 days of age. Following inoculation at 10 days of age this higher temperature was maintained for a period of 3 to 4 days, the normal incubation period for fowl typhoid.

Fever temperatures of 1 to 2 C above normal developed in susceptible individuals during the later stages of the disease. Within both resistant and susceptible strains a fever at this time was associated with susceptibility.

Artificially reduced body temperatures lowered resistance in all strains below that of corresponding controls. Resistant chicks were more nearly able to maintain normal body temperature under adverse conditions indicating a more efficient temperature-regulating mechanism.

Phagocytosis studies in vitro revealed no differences in the phagocytic abilities of polymorphonuclear leucocytes from resistant and susceptible strains. Both strains showed greater phagocytosis with increasing temperature. This suggests that phagocytosis in vivo would occur at a faster rate in resistant chicks due to their higher body temperature.

Little intracellular digestion of phagocytosed bacteria occurred in polymorphonuclear leucocytes from the sus-

ceptible strain, while marked lysis of bacteria was evidenced by resistant cells. This qualitative difference in intracellular enzymes could account for specific disease resistance without imparting a generalized resistance.

Bacteridal properties of whole blood or serum are not a factor in fowl typhoid resistance.

The ability of polymorphonuclear leucocytes to digest phagocytosed bacteria appears to be a major factor in genetic resistance to *Salmonella gallinarum*. Differences in body temperature assume an important supporting role.

REFERENCES

Bell. A. E. 1947, Temperature changes in the chick during the course of fowl typhoid. Iowa Acad. Sci. Proc. **54:** 303–308.

Bordet, J. 1897, Contribution a l'étude du sérum antistreptoccique. Ann. Inst. Pasteur, **11:** 177–213.

Cottingham, E. and Mills, C. A. 1943, Influence of environmental temperature and vitamin-deficiency upon phagocytic functions. J. Immunol. **47:** 493–502.

Fronda, F. M. 1921, A comparative study of the body temperature of the different species and some representative breeds of poultry. A preliminary report. Poul. Sci. **1:** 16–22.

Gowen, J. W. 1937–1947, Breeding for resistance to fowl typhoid in poultry. Rept. Agr. Res. Iowa Agr. Exp. Sta. 1937; 157–158; 1938, 150–151; 1939: 155–156; 1940: 190–192; 1941: 170–171; 1942: 187–189; 1943: 176–178; 1944: 202–204; 1945: 275–278; 1946: 254–257; 1947: In press.

———. 1948, Inheritance of immunity in animals. Ann. Rev. Microbiol. **2:** 215–255.

Gowen, J. W. and Calhoun, M. L. 1943, Factors affecting genetic resistance of mice to mouse typhoid. J. Infect. Dis. **73:** 40–56.

Hamburger, H. J. 1912, Physikalisch-chemische Untersuchungen über Phagozyten, Wiesbaden, J. F. Bergman, p. 247.

Harmon, D. R., Zarafonetis, C. and Clark, P. F. 1946, Temperature relations in phagocytosis. J. Bact. **52:** 337–347.

Lambert, W. V. 1930, Breeding for resistance to fowl typhoid in poultry. Rep. Agr. Res., Iowa Agr. Exp. Sta. 58–59.

———. 1932, Natural resistance to disease in the chick. I. The effect of selective breeding on natural resistance to fowl typhoid. II. Bacteriological studies upon surviving birds of the resistant stock in relation to progeny resistance. III. The comparative resistance of different breeds. J. Immunol. **23:** 229–260.

Lambert, W. V. and Knox, C. W. 1928, The inheritance of resistance to fowl typhoid in chickens. Iowa State College Jour. Sci. **2:** 179–187.

———. 1932, Selection for resistance to fowl typhoid in the chicken with reference to its inheritance. Iowa Agr. Exp. Sta. Res. Bull. 153.

Lamoreux, W. F. and Hutt, F. B. 1939, Variability of body temperature in the normal chick. Poul. Sci. **18:** 70–75.

Ledingham, J. C. G. 1908, The influence of temperature on phagocytosis. Proc. Roy. Soc., London, s. B. **80:** 188–195.

Madsen, T. and Wulff, O. 1919, Influence de la température sur la phagocytose. Ann. Inst. Pasteur, **33:** 437–447.

Metchnikoff, E. 1907, Immunity in Infective Diseases, Cambridge, University Press, pp. 128–324.

Mudd, S., Lucke, B. and Strumia, M. 1933, The effect of flagellar and somatic sensitization of the typhoid bacillus on phagocytosis by macrophages and polymorphonuclear leucocytes—Intracellular digestion. J. Immunol. **24:** 493–504.

Oakberg, E. F. 1946, Constitution of liver and spleen as a physical basis for genetic resistance to mouse typhoid. J. Infect. Dis. **78:** 79–98.

Pasteur, L., Jaubert, J. and Chamberland, C. 1878. La théorie des germes et ses applications à la médicine et à la chirurgie. Bull. Acad. med., 2d series, **7:** 432–453.

Rous, P. and Jones, F. S. 1916, The protection of pathogenic microorganisms by living tissue cells. J. Exper. Med. **23:** 601–612.

Schnetzler, E. E. and Hartsell, S. E. 1940, Normal bactericidins in sera of the domestic fowl. Poul. Sci. **19:** 431–434.

Scholes, J. C. and Hutt, F. B. 1942, The relationship between body temperature and genetic resistance to *Salmonella pullorum* in the fowl. N. Y. (Cornell) Agr. Exp. Sta. Memoir 244.

Severens, J. M., Roberts, E. and Card, L. E. 1944, A study of the defense mechanism involved in hereditary resistance to pullorum disease of the domestic fowl. J. Infect. Dis. **75:** 33–46.

Wagner, K. E. 1890, Contribution à l'étude de l'immunité. Le charbon des poules. Ann. Inst. Pasteur, **4:** 570–602.

Werigo, M., 1894, Développement du charbon chez le lapin. Ann. Inst. Pasteur, **8:** 1–53.

RELATIVE SUSCEPTIBILITY OF *AËDES AEGYPTI, AËDES ALBOPICTUS, AËDES CANADENSIS* AND *ANOPHELES QUADRIMACULATUS* TO *PLASMODIUM GALLINACEUM*

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Although there is a large amount of information on the susceptibility of mosquitoes to infection with various malaria parasites, much of it is only semi-quantitative. Some workers, however, have fed mosquitoes of two species on the same infected animal at the same time and compared the infections resulting in the mosquitoes. For example, Trembley[1] found that *Aëdes aegypti* was less susceptible to *Plasmodium gallinaceum* than *Aëdes atropalpus*. This procedure eliminates an important variable, the degree of infectiousness of the blood, which was found to change rapidly by Lumsden and Bertram[2] and Cantrell and Jordan.[3] In the present report we have compared three species of mosquito with *A. aegypti* after feeding them simultaneously on the same chicken infected with *P. gallinaceum*. A comparison was also made of the size attained by the oöcysts after 5 days in *A. aegypti* and *Aëdes albopictus*.

A. aegypti *and* A. albopictus

Two-day old mosquitoes of each species which had not taken any food were allowed to engorge on a chicken in the rising phase of parasitemia when 12% of the red blood cells were infected and 2.2% of the parasites were gametocytes. Under light ether anesthesia the mosquitoes were examined and those that had not fully engorged were discarded. After 120 hours incubation at 29 C the mosquitoes were dissected and oöcysts counted and measured. Mean oöcyst diameters were obtained by measuring at least 30 oöcysts from each mosquito, except in 5 instances, when from 14 to 28 were measured, and then calculating from these means the mean diameter for each species. This was done because the mean diameter sometimes varies significantly from mosquito to mosquito as shown in table 2 and as reported for *Plasmodium cathemerium* and *Plasmodium relictum* in *Culex pipiens* by Huff.[4]

As may be seen in table 1, the difference between the mean number of oöcysts in *A. aegypti* and *A. albopictus*, 95 ± 64, has a probability of 0.13, which cannot be taken as evidence of a real difference between these species. The difference in oöcyst diameter, $4.7 \pm 1.33\mu$ has a probability of 0.0009 and can be considered highly significant. In this connection it should be pointed out that a valid comparison of oöcyst diameters can be made only when the mosquitoes are allowed to feed from the same infected chicken, as we have found that the source of the infecting blood meal affects the size of oöcysts as well as the number of oöcysts.[3] Furthermore, we found a significant negative correlation

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1. Trembley, H. L. 1946, J. Parasitol. **32**: 499–501.
2. Lumsden, W. H. R. and Bertram, D. S. 1940, Ann. Trop. Med. **34**: 135–160.
3. Cantrell, W. and Jordan, H. B. 1946, J. Infect. Dis. **78**: 153–159.
4. Huff, C. G. 1940, Am. J. Hyg. **32** (Sec. C): 71–80.

between the size of oöcysts and intensity of infection with *P. gallinaceum* in *A. aegypti* ($r = -0.57$ with $P < 0.001$) and a suggestive one in *A. albopictus* ($r = -0.39$ with $P = 0.02$). The t test of the significance of the deviation of the coefficient of correlation from 0 was used to evaluate these figures. On the other hand, Huff[5] did not find such a correlation with *P. cathemerium* and *P. relictum* in *C. pipiens*.

TABLE 1.—*Relative susceptibility to* P. gallinaceum *of* A. albopictus, A. canadensis, *and* A. quadrimaculatis *as compared with* A. aegypti.

	Number dissected	Number infected	Oöcyst per infected mosquito		Oöcyst diameter in μ	
			Mean	Range	Mean	Range
A. aegypti	20	20	373 ± 44*	62–800	37 9 ± 0.85	31.9–46.5
A. albopictus	21	20	278 ± 47	0–605	33.2 ± 1.01	25.6–41.8
A. aegypti	25	20	24 ± 7.6	0– 76		
A. canadensis	23	2	1	0– 1		
A. aegypti	18	17	88 ± 9.6	0–176		
A. quadrimaculatus	17	9	45 ± 26	0–220		

* Standard error.

TABLE 2.—*Size of oöcysts of* P. gallinaceum *in* A. aegypti *and* A. albopictus *after 120 hours at 29 C.*

A. aegypti			*A. albopictus*		
Mosquito no.	Mean oöcyst diameter	Range of diameter*	Mosquito no.	Mean oöcyst diameter	Range of diameter
1	33.7 ± 1.03†	17–50	1	31.9 ± 0.75	17–46
2	33.4 ± 2.11	17–50	2	28.2 ± 1.68	13–41
3	36.7 ± 0.73	23–46	3	29.9 ± 0.66	17–46
4	45.1 ± 1.71	18–55	4	30.0 ± 0.73	17–36
5	45.0 ± 1.74	20–58	5	35.0 ± 1.33	17–46
6	41.7 ± 1.04	17–51	6	37.0 ± 0.81	25–46
7	35.3 ± 0.63	25–46	7	31.9 ± 0.78	18–41
8	41.0 ± 1.41	17–55	8	36.7 ± 0.95	30–41
9	38.7 ± 1.46	23–48	9	41.8 ± 1.43	17–50
10	32.0 ± 1.33	17–46	10	37.7 ± 1.01	25–50
11	36.7 ± 0.98	23–46	11	25.6 ± 0.60	20–33
12	38.3 ± 1.14	17–50	12	32.2 ± 0.75	25–46
13	38.0 ± 1.03	17–50	13	34.2 ± 1.54	17–50
14	36.3 ± 1.16	17–50	14	32.4 ± 1.29	17–41
15	46.5 ± 1.28	28–51	15	33.7 ± 0.85	17–46
16	35.7 ± 1.49	25–46	16	31.2 ± 0.85	21–50
17	31.9 ± 1.54	17–50	17	31.0 ± 0.96	25–41
18	34.0 ± 1.00	17–50	18	41.3 ± 0.98	33–45
19	39.3 ± 1.29	17–58	19	29.4 ± 1.84	13–45
20	39.7 ± 1.87	17–53	20	33.7 ± 1.04	17–41

* In μ.
† Standard error.

A. aegypti *and* A. canadensis

Groups of these species were fed on an infected chicken with the results shown in table 1. Only 2 of 23 *A. canadensis* were infected with one oöcyst each, while 20 of 25 *A. aegypti* were infected with an average of 24 ± 7.6 oöcysts.

Another group of *A. canadensis* were fed and after 9 days at 28 C 25 were dissected. Three had sporozoites in their salivary glands and one had one oöcyst on the midgut.

A. aegypti *and* Anopheles quadrimaculatus

Groups of these species were fed on a chicken when 17% of the red blood cells were parasitized and about 0.3% of the parasites were gametocytes. After 6 days incubation at 29 C they were dissected with the results shown in table 1. Seventeen of 18 *A. aegypti* and 9 of 17 *A. quadrimaculatus* were infected. The probability of this differ-

5. Huff, C. G. 1941, Am. J. Hyg. **34** (Sec. C): 18–21.

ence is less than 0.0015 calculated by expansion of the binomial theorem. As to the intensity of the infection, *A. aegypti* had 88 ± 9.6 oöcysts per infected mosquito, while *A. quadrimaculatus* had 45 ± 26 oöcysts per infected mosquito. The 9 infected *A. quadrimaculatus* had 1, 2, 6, 8, 10, 10, 11, 134 and 220 oöcysts, respectively, which gave a value of about 0.6 for P by Student's t test. Although this figure indicates no interspecific difference, the sample was too small to warrant any conclusion.

SUMMARY

1. *Aëdes aegypti* and *Aëdes albopictus* were highly susceptible to infection with *Plasmodium gallinaceum*. The oöcysts developing in *A. aegypti* for 120 hours at 29 C attained a significantly larger size ($37.9 \pm 0.85\mu$) than those in *A. albopictus* (33.2 ± 1.01).

2. *A. aegypti* was more susceptible than *Aëdes canadensis*.

3. In a comparison of *A. aegypti* and *Anopheles quadrimaculatus* a significantly higher proportion of *A. aegypti* became infected. The number of oöcysts in the infected *Anopheles* was so variable an adequate comparison of the species in this respect could not be made.

4. The susceptibility of *A. quadrimaculatus* to *P. gallinaceum* previously reported by Haas and Akins[6] was confirmed.

5. The susceptibility of *A. canadensis* to *P. gallinaceum* is reported for the first time.

6. The size of oöcysts was found to be negatively correlated with the intensity of the infection in *A. aegypti*. ($r = -0.57$ with $P < 0.001$) and probably in *A. albopictus* ($r = -0.39$ with $P = 0.02$).

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6. Haas, V. H. and Akins, H. 1947, J. Nat. Malaria Soc. **6**: 244–245.

NUTRITION AND POLIOMYELITIS. THE EFFECTS OF DEFICIENCIES OF PHOSPHORUS, CALCIUM, AND VITAMIN D ON THE RESPONSE OF MICE TO THE LANSING STRAIN OF POLIOMYELITIS VIRUS

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Since it was first reported[1] that a dietary deficiency of vitamin B_1 decreases the susceptibility of mice to the murine-adapted Lansing strain of poliomyelitis virus, the effects of several other dietary deficiencies have been studied. Recently, Lichstein et al[2] have reported that a deficiency of phosphorus increased the resistance of mice to Thelier's virus to an extent comparable to the protection given by a deficiency of vitamin B_1 against the Lansing strain of poliomyelitis virus. A deficiency of calcium, on the other hand, gave no protection. At the time the studies mentioned above were published, the present authors had completed a study (first series) on the effect of a deficiency of phosphorus on the resistance of mice to the Lansing strain of the virus of poliomyelitis. The diet was also deficient in vitamin D, and to the low-phosphorus diets of some of the groups of animals beryllium carbonate was added to make the phosphorus deficiency more severe. In none of the experiments was there any evidence that the deficiency protected the mice against the virus. The mice were markedly deficient, as shown not only by general weight loss and several deaths among the controls, but also by the levels of inorganic phosphate in the blood. In numerous cases the inorganic phosphorus of the blood of sacrificed animals was found to be below 2 mg per 100 ml of blood, as contrasted to 6 mg or more in normal animals of corresponding age.

In regard to the difference in the viruses used by Lichstein et al[2] and the present authors, it has been shown by the Wisconsin workers that some deficiencies increase the resistance of mice against the Lansing strain but not against Theiler's virus,[3] while with other deficiencies the reverse is true.[4] Apparently the variations in the responses to a deficiency of phosphorus in the host is another example of this type of virus specificity. There is also suggestive evidence that the presence or absence of vitamin D might influence the results in an indirect way. Jones[5] has

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1. Foster, C., Jones, J. H., Henle, W. and Dorfman, F. 1942, Response to murine poliomyelitis virus (Lansing strain) of mice on different levels of thiamine intake. Proc. Soc. Exper. Biol. & Med. **51**: 215.
2. Lichstein, H. C., McCall, K. B., Kearney, E. B., Elvehjem, C. A. and Clark, P. F. 1946, Effect of minerals on susceptibility of Swiss mice to Theiler's virus. Proc. Soc. Exper. Biol. & Med. **62**: 279.
3. Rasmussen, A. F., Jr., Waisman, H. A. and Lichstein, H. C. 1944, Influence of riboflavin on susceptibility of mice to experimental poliomyelitis. Proc. Soc. Exper. Biol. & Med. **57**: 92.
4. Lichstein, H. C., Waisman, H. A., Elvehjem, C. A. and Clark, P. F. 1944, Influence of pantothenic acid deficiency on resistance of mice to experimental poliomyelitis. Proc. Soc. Exper. Biol. & Med. **56**: 3.
5. Jones, J. H. 1944, The production of hyper-

shown that on a diet very low in phosphorus and containing a liberal supply of calcium, a hypercalcemia is produced in the rat by therapeutic levels of vitamin D. With increasing amounts of calcium in the diet, the hypercalcemia becomes more pronounced. It was thought that in the experiments of Lichstein and associates[2] the protection on the diets low in phosphorus and containing resistant, were actually more susceptible to the Lansing strain of the virus than were the controls on the complete diet. In view of these unexpected results, the number of groups of animals was increased, and the experiment, in the enlarged form, was repeated twice (experiments II and III). These experiments gave results similar to the first. In the present paper, the experiments

TABLE 1.—*Composition of diets in g per 100 g.*

Diet no.	1	2	3	4	5	6	7	8	9	10
Cerelose	71.5	72.1	73.5	72.1	73.5	71.0	71.0	72.5	72.5	71.5
Fibrin (alcohol extracted)	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0
Cellufour	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Linseed oil	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Wheat germ oil	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Carotine-in-oil*	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)	(5.0)
Drisdol†	(0.4)	(0.4)	(0.4)	—	—	(0.4)	—	(0.4)	—	—
Salt 12‡	4.0	—	—	—	—	—	—	—	—	4.0
Salt 20§	—	2.0	2.0	2.0	2.0	2.0	2.0	—	—	—
Salt 21‖	—	—	—	—	—	—	—	3.0	3.0	—
$CaCO_3$	—	1.4	—	1.4	—	2.5	2.5	—	—	—
Relative amounts of P, Ca, and vitamin D in diets¶	MP, MCa, D+	LP, MCa, D+	LP, LCa, D+	LP, MCa, D−	LP, LCa, D−	LP, HCa, D+	LP, HCa, D−	MP, LCa, D+	MP, LCa, D−	MP, MCa, D−

* Wyeth Inc. 275 USP units per drop.
† Winthrop Chemical Co., Inc., Calciferol in propyline glycol. 250 USP units per drop.
‡ Jones, J. H. and Foster, C., 1942, J. Nutrition, **24**: 245.
§ Salt mixture free from phosphorus and calcium. Same as Salt 12 except KH_2FO_4 of latter is replaced by an equimolecular amount of KCl, the CaO_3 is omitted, and the $MgSO_4.7\ H_2O$ is replaced by $MgCO_3$.
‖ Salt mixture free from calcium. Same as Salt 12 except $CaCO_3$ is omitted and the KH_2PO_4 is replaced by an equimolecular amount of K_2HPO_4.
The following synthetic B vitamins were added, expressed in mg per 100 g of diet: thiamine chloride 0.5, riboflavin 0.5, pyridoxine 0.5, calcium pantothenate 5.0, nicotinic acid 10.0, inositol 10.0, p-aminobenzoic acid 10.0, choline chloride 200.
¶ L = low, M = medium, H = high, + = vitamin D added, − = no vitamin D added.

vitamin D may have been due to a hypercalcemia and the toxicity which usually results from the high serum calcium.

In order to explore the possible effects of dietary levels of phosphorus, calcium, and vitamin D on the response of the Lansing strain of poliomyelitis virus, another series of experiments (second series) was carried out. In these experiments, in addition to a deficiency of phosphorus, the effects of various dietary levels and combinations of phosphorus, calcium, and vitamin D were studied.

Experiment I, second series, indicated that on some of these deficient diets the mice, instead of being more

calcemia with small amounts of vitamin D. J. Nutrition, **28**: 7.

of the first series, completed before the appearance of the Wisconsin publication, are reviewed briefly, and the results of experiment III of the second series on the various combinations of dietary levels of phosphorus, calcium, and vitamin D are presented in detail.

EXPERIMENTAL

The mice used in both series of experiments were raised in this laboratory on a modified Steenbock stock diet[6] containing vitamin D. The pre-weaning and experimental regimen of the animals in the two series differed but slightly. The following refers to the second series. On the 18th day after the young were born the vitamin D was removed from the stock diet. The young were

6. Steenbock, H. 1923, A satisfactory ration for stock rats. Science, **58**: 449.

weaned at 23 days of age. In each of experiments II and III, at ages varying from 23 to 28 days, with the great majority between 25 and 27 days, the animals were divided into 20 groups, placed in individual cages, and given the complete diet minus vitamin D (diet 10, table 1). Two days later, two groups were put on each of the 10 diets, the compositions of which are given in table 1. These diets, before any salts are added, contain approximately 0.02% phosphorus and not over 0.03% calcium. At the bottom of each column of dietary constituents is shown whether the diet is low or medium in phosphorus; low, medium, or high in calcium; and whether or not the diet contains added vitamin D. The relative amounts of phosphorus and calcium are indicated by the letters L, M, and H, and the presence of absence of vitamin D is shown by a plus or minus sign. For example, diet 2 is low in phosphorus, contains a medium amount of calcium, and vitamin D has been added. No phosphorus or calcium was added to the diets low in these elements; the medium phosphorus diets contain about 0.33% phosphorus, and the medium and high calcium diets about 0.56% and 1.0% calcium, respectively.

The animals were weighed every day and were housed and cared for as described in previous publications.[7,8] When the animals on the phosphorus-low diets showed signs of a deficiency, at least two animals on each diet were given a small dose of heparin and bled under light ether anesthesia for phosphorus determination. The inorganic phosphorus was determined on 0.2 ml of whole blood from each animal by a modification of Gomori's method.[9] In experiment II the levels of phosphorus were considerably below normal although the blood level did not always parallel the other signs of deficiency. In experiment III, however, the blood phosphorus of all 12 samples taken from animals on the diets low in phosphorus gave values from 1.6 to 4.2 mg percent of inorganic phosphorus, with only one value above 3.5 and an average of 2.5. On the 18th day in experiment II, and on the 25th day in experiment III, after the initial feeding of the experimental diets, the mice in one group on each diet were inoculated intracerebrally with a mouse-brain suspension containing the virus; and the animals in the remaining group on each diet were injected with an uninfected mouse-brain suspension. Beginning the day following the inoculations, each infected animal was removed from its cage and examined every 8 hours for signs of poliomyelitis. Lichstein et al[2] have stated that it is difficult to distinguish between the paralysis of Theiler's virus and that due to a severe deficiency of phosphorus. The present authors, working with the murine-adapted strain of poliomyelitis virus, have experienced this trouble in only a small percentage of the total cases. In the first place, the paralysis of phosphorus deficiency is invariably in the hind legs. In no instance was the paralysis observed in the front legs of any of the animals in the phosphorus-low groups which did not receive virus. On the other hand, the paralysis of poliomyelitis occurs much more frequently in the front than in the hind legs, and seldom is paralysis seen in either hind leg without its also being observed in one or both front legs if the animals are examined every 8 hours. In addition, there are other signs of poliomyelitis which never result from a phosphorus deficiency, such as paralysis of the neck and conjunctival exudate. This conjunctival exudate has never been seen in this laboratory except in animals which had been inoculated with the Lansing strain of poliomyelitis virus. In very few cases, however, was this the only sign of poliomyelitis observed. By examining the animals every 8 hours and watching for the above signs, over 80% of the inoculated animals which died were seen to have one or more of the above signs of poliomyelitis. There were very few deaths in the uninfected control groups, and these came near the end of the experimental periods after most of the deaths from poliomyelitis had occurred. The experiments were discontinued 28 days after the animals had been inoculated.

7. Foster, C., Jones, J. H., Henle, W. and Dorfman, F. 1944, The effect of vitamin B_1 deficiency and of restricted food intake on the response of mice to the Lansing strain of poliomyelitis virus. J. Exper. Med. **79**: 221.
8. Foster, C., Jones, J. H., Henle, W. and Dorfman, F. 1944, The comparative effects of vitamin B_1 deficiency and restriction of food intake on the response of mice to the Lansing strain of poliomyelitis virus, as determined by the paired feeding technique, J. Exper. Med. **80**: 257.
9. Gomori, G. 1941–42, A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. & Clin. Med. **27**: 955.

RESULTS

In none of the experiments of the first series was there any evidence that the combined deficiency of phosphorus and vitamin D protected the mice to any degree against the virus, although the animals were in a state of advanced

deficiency. In fact, in most cases, the deficient animals were more susceptible than the controls on the complete diets. In only a few instances was the difference in susceptibility statistically significant, and then for only a few days of the experiment.

The results of the second and third experiments of the second series were so similar that only those of experiment III are presented. In table 2, the cumulative deaths, expressed in percent of total number of animals, are given at two-day intervals.

uninfected brain, and these occurred in the later part of the experiment. In the virus-inoculated animals, however, the proportion of deaths without signs of poliomyelitis to the total deaths was highest in the first part of the experiment. Furthermore, most of these latter animals died without showing severe signs of malnutrition; and since they were being examined every 8 hours, it is not likely that they died of nutritional deficiency. It appears that on some of the deficient diets studied in this experiment, the infected animals died

TABLE 2.—*Deaths in cumulative percent of virus-injected mice, series II, experiment III.*

Days after inoculation	Group number 1	2	3	4	5	6	7	8	9	10
	Number of mice 41	39	41	39	41	38	35	39	40	41
2	—	—	5	10	15	3	6	3	—	—
4	2	3	10	18	17	8	20	5	3	2
6	5	8	15	18	29	11	26	10	8	2
8	5	13	32	31	51	24	29	18	13	5
10	22	28	51	59	66	39	43	31	25	20
12	39	41	63	79	76	61	66	49	38	32
14	51	49	78	85	85	74	80	64	55	51
16	59	64	85	90	88	79	83	79	68	61
18	61	72	88	90	88	89	89	82	73	66
20	66	79	93	90	88	89	89	90	75	71
22	66	79	93	92	90	95	89	90	75	73
24	68	82	93	92	93	95	89	92	80	73
26	68	82	93	95	93	95	89	92	83	73
28	68	82	93	95	95	96	89	92	85	76

Microtype figures in body of table are cumulative percent of mice which died without signs of polio being observed.

The animals which died on the day of inoculation have been excluded both from the total number and from the number that died. The numbers in small type in each column indicate the percentage of animals which died without any signs of poliomyelitis having been observed. These numbers, for the most part, are greater in the groups on the diets deficient in two or more of the factors under consideration than in the groups on the less deficient diets. Even though no signs of poliomyelitis were observed on the 8-hourly inspections, there is evidence that the great majority of these deaths was due to poliomyelitis. In the first place, there were very few deaths among the animals on the same diets injected with the suspension of

sooner after the appearance of the first signs of poliomyelitis than on any of the other deficient diets previously studied in this laboratory. In view of these facts, an assumption has been made which must be largely, if not entirely, correct—namely, that all deaths in the infected groups occurring from the second day after inoculation on were due to poliomyelitis.

Table 2 shows that the death rate during the first few days was higher in nearly all of the groups on the deficient diets than in the group on the complete diet (group 1), although, as previously stated, there were practically no deaths among the animals in the control groups on similar diets but which were injected with normal brain suspension.

Group 5, for example, was on the diet low in phosphorus and calcium and with no added vitamin D. Deaths in this group were very high as they also were in groups 4 and 7, both of which diets are low in phosphorus and contain no vitamin D. The higher death rate, however, was not due entirely to the absence of vitamin D; for in group 3 deaths were rather high, and the diet of this group contains the vitamin but is low in phosphorus and calcium. Likewise, the high death rate was not due entirely to the low phosphorus; for in group 2 in which the phosphorus is low but the calcium is medium and vitamin D has been added, the death rate was only slightly higher (difference not significant) than that in the group on the complete diet (diet 1). For the most part, the highest death rate in the early part of the experiment was on those diets which are most deficient. The highest death rate during that period was on the diet low in phosphorus and calcium and containing no vitamin D. The next highest was on the diet low in phosphorus and calcium but containing vitamin D.

The animals on the complete diet (diet 1) were given sufficient virus to kill from about 70 to 80%. The highest death rate of these animals was usually between the 10th and 15th days after inoculation. It was before this period that the difference in the number of deaths on the deficient diets compared to the complete diets was greatest, but on several diets a definite difference was maintained to the end of the experiment.

Each inoculated group was compared statistically (chi-square tests) with every other inoculated group for every day of the experiment. The comparisons for experiment III are presented in pairs in table 3. Only those pairs in which a statistically significant difference (chi-square of 3.84 or more) occurred on one or more days of the experiment are presented. On those days in which a significant difference did occur, the percentage deaths in the two groups are listed in the proper day column. If the chi-square for the particular day in any comparison is from 3.84 to 5.9, the percentage number appears without further identification; if the chi-square is from 6.0 to 9.0, the percentage number is italicized; and if the chi-square is 9.1 or greater, the percentage number is marked with an asterisk. By referring to table 3, it is seen that no comparison for groups 1 (complete diet) and 2 (low phosphorus diet) is given. This indicates that on no day of the experiment was there a statistically significant difference in cumulative deaths between these two groups. The first pair given is a comparison between group 1 and group 3 (diet low in phosphorus and calcium); and, as table 3 shows, there was a significant difference on many days of the experiment, and this difference was of a high order.

As a whole, table 3 shows that on several of the deficient diets, the death rate was significantly greater than on the control diet (group 1). In some cases the significance was of a high order, and in several groups the difference remained significant to the end of the experiment. It is interesting to note that the death rate was not significantly greater on the diets low in either phosphorus or vitamin D than on the complete diet. In contrast, on the diet low only in calcium the death rate was considerably greater than on the complete diet. This difference was highly significant for the last week of the experiment.

It can also be observed that there were significant differences among the various groups of animals on the deficient diets.

In the introduction to this paper, it

TABLE 3.—*Significant differences in mortality percent, series II, experiment III.*

No.	Diet	Group	Days after inoculation																										
			2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
41	MP, MCa, D+	1	.	.	.	.	.	.	5	20	22	.	39	46	51	56	59	61	61	63	66	66	66	68	68	68	68	68	68
41	LP, LCa, D+	3	.	.	.	.	.	.	32*	44	*51*	.	63	71	*78*	*85*	*85*	*85*	*88*	*88*	*93*	*93*	*93*	*93*	*93*	*93*	*93*	*93*	*93*
41	MP, MCa, D+	1	0	*2*	*2*	.	.	5	5	.	*22*	32	39	46	51	56	59	61	61	63	66	66	66	68	68	68	68	68	68
39	LP, MCa, D−	4	10	18	18	.	.	21	31*	.	59*	*64*	79*	79*	85*	*85*	*90*	*90*	*90*	*90*	*90*	*92*	*92*	*92*	*92*	*92*	95*	95*	95*
41	MP, MCa, D+	1	0	*2*	*2*	5	5	5	5	20	*22*	32	39	46	51	56	59	61	61	63	66	66	66	68	68	68	68	68	68
41	LP, LCa, D−	5	*15*	17	17	*24*	*29*	37*	51*	59*	66*	71*	76*	80*	85*	*85*	*88*	*88*	*88*	*88*	88	88	*90*	*93*	*93*	*93*	*93*	95*	95*
41	MP, MCa, D+	1	.	.	.	.	.	.	5	.	.	32	.	46	51	56	.	61	61	63	66	66	66	68	68	68	68	68	68
38	LP, HCa, D+	6	.	.	.	.	.	.	24	.	.	55	.	68	74	79	.	*87*	*89*	*89*	*89*	*89*	95*	*95*	*95*	*95*	*95*	*95*	*95*
41	MP, MCa, D+	1	.	*2*	*2*	5	5	5	5	.	.	32	39	46	51	56	59	61	61	63	66	66	66	68	68	68	68	68	68
35	LP, HCa, D−	7	.	17	*20*	*23*	*26*	*29*	*29*	.	.	57	66	71	*80*	80	83	83	*89*	*89*	89	89	89	89	89	89	89	89	89
41	MP, MCa, D+	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	59	.	61	63	66	66	66	68	68	68	68	68	68
39	MP, LCa, D+	8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	79	.	82	85	*90*	*90*	*90*	90	*92*	*92*	*92*	*92*	*92*
39	LP, MCa, D+	2	.	.	.	.	.	.	.	.	28	.	41	46	49	51	64	.	.	.	.	.	.	.	.	.	.	.	.
41	LP, LCa, D+	3	.	.	.	.	.	.	.	.	51	.	63	71	*78*	85*	85	.	.	.	.	.	.	.	.	.	.	.	.
39	LP, MCa, D+	2	0	*3*	*3*	.	.	.	.	.	28	33	41	46	49	51	64	72	72	72	.	.	.	.	.	.	.	.	.
39	LP, MCa, D−	4	10	18	18	.	.	.	.	.	*59*	*64*	79*	79*	85*	85*	*90*	90	90	90	.	.	.	.	.	.	.	.	.
39	LP, MCa, D+	2	0	*3*	*3*	5	8	8	13	23	28	33	41	46	49	51	64	.	.	.	.	.	.	.	.	.	.	.	.
41	LP, LCa, D−	5	*15*	17	17	*24*	*29*	37*	51*	59*	66*	71*	76*	80*	85*	85*	*88*	.	.	.	.	.	.	.	.	.	.	.	.
39	LP, MCa, D+	2	.	.	.	.	.	.	.	.	.	.	.	.	49	51	.	.	72	72	.	.	79	.	.	.	.	.	.
38	LP, HCa, D+	6	.	.	.	.	.	.	.	.	.	.	.	.	74	*79*	.	.	89	89	.	.	95	.	.	.	.	.	.
39	LP, MCa, D+	2	.	*3*	*3*	5	8	8	.	.	.	33	41	46	49	51	.	.	.	.	.	.	.	.	.	.	.	.	.
35	LP, HCa, D−	7	.	17	20	23	26	29	.	.	.	57	66	71	*80*	*80*	.	.	.	.	.	.	.	.	.	.	.	.	.
41	LP, LCa, D+	3	.	.	.	.	.	.	32	44	*51*	.	63	.	78	*85*	.	.	.	.	93	93	93	.	.	.	.	.	.
40	MP, LCa, D−	9	.	.	.	.	.	.	13	20	25	.	38	.	55	60	.	.	.	.	75	75	75	.	.	.	.	.	.
41	LP, LCa, D+	3	.	.	.	.	.	*20*	32*	44*	*51*	*51*	*63*	*71*	*78*	*85*	*85*	85	88	88	*93*	93	93	93	93	93	93	93	93
41	MP, MCa, D−	10	.	.	.	.	.	2	5	10	20	24	32	44	51	59	61	63	66	68	71	73	73	73	73	73	73	73	76
39	LP, MCa, D−	4	.	.	.	.	.	.	.	.	*59*	*64*	*79*	79	85	.	.	.	.	.	.	.	.	.	.	.	.	.	.
39	MP, LCa, D+	8	.	.	.	.	.	.	.	.	31	36	49	54	64	.	.	.	.	.	.	.	.	.	.	.	.	.	.
39	LP, MCa, D−	4	10	18	18	18	.	.	.	38	59*	*64*	79*	*79*	*85*	*85*	90	90	90	.	.	92	92	92	.	.	.	.	.
40	MP, LCa, D−	9	0	3	3	3	.	.	.	20	25	33	38	50	55	60	68	73	73	.	.	75	75	78	.	.	.	.	.
39	LP, MCa, D−	4	10	18	18	18	18	*21*	31*	*38*	59*	64*	79*	79*	85*	*85*	*90*	*90*	*90*	90	90	92	92	92	92	92	*95*	*95*	95
41	MP, MCa, D−	10	0	2	2	2	2	2	5	10	20	24	*32*	44	51	59	61	63	66	68	71	73	73	73	73	73	73	73	76
41	LP, LCa, D−	5	.	.	.	24	29	37	*51*	59	66	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
38	LP, HCa, D+	6	.	.	.	8	11	13	24	*32*	39	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
41	LP, LCa, D−	5	.	.	.	.	.	.	51	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
35	LP, HCa, D−	7	.	.	.	.	.	.	29	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
41	LP, LCa, D−	5	.	.	.	24	29	.	51*	*59*	66*	71*	*76*	*80*	85	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
39	MP, LCa, D+	8	.	.	.	8	10	.	18	26	31	36	49	54	64	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
41	LP, LCa, D−	5	*15*	17	17	*24*	*29*	*37*	51*	59*	66*	71*	76*	*80*	85*	*85*	88	.	.	.	.	.	.	.	.	.	.	.	.
40	MP, LCa, D−	9	0	3	3	3	8	10	13	20	25	33	38	50	55	60	68	.	.	.	.	.	.	.	.	.	.	.	.
41	LP, LCa, D−	5	*15*	17	17	*24*	29*	37*	51*	59*	66*	71*	76*	80*	*85*	*85*	*88*	*88*	88	88	.	.	90	93	93	93	93	*95*	*95*
41	MP, MCa, D−	10	0	2	2	2	2	2	5	10	20	24	32	44	51	59	61	63	66	68	.	.	73	73	73	73	73	73	76
38	LP, HCa, D+	6	.	.	.	.	.	.	.	.	.	55	61	.	.	.	.	.	.	.	.	.	95	95	.	.	.	.	.
40	MP, LCa, D−	9	.	.	.	.	.	.	.	.	.	33	38	.	.	.	.	.	.	.	.	.	75	78	.	.	.	.	.
38	LP, HCa, D+	6	.	.	.	.	.	.	24	32	.	*55*	*61*	68	.	.	.	87	*89*	89	89	89	*95*	*95*	*95*	*95*	*95*	*95*	*95*
41	MP, MCa, D−	10	.	.	.	.	.	.	5	10	.	24	*32*	44	.	.	.	63	66	68	71	73	73	73	73	73	73	73	76
35	LP, HCa, D−	7	.	17	*20*	*23*	26	29	.	.	.	57	*66*	.	80	.	.	.	.	.	.	.	.	.	.	.	.	.	.
40	MP, LCa, D−	9	.	*3*	*3*	*3*	8	10	.	.	.	33	38	.	55	.	.	.	.	.	.	.	.	.	.	.	.	.	.
35	LP, HCa, D−	7	.	17	*20*	*23*	*26*	29*	*29*	*37*	43	*57*	*66*	71	*80*	80	83	.	89	89	.	.	.	.	.	.	.	.	.
41	MP, MCa, D−	10	.	2	2	2	2	2	5	10	20	24	*32*	44	51	59	61	.	66	68	.	.	.	.	.	.	.	.	.
39	MP, LCa, D+	8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	90	.	.	.	92	92	92	92	92
41	MP, MCa, D−	10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	71	.	.	.	73	73	73	73	76

If the chi-square is from 3.84 to 5.9, the percentage number appears in regular type.
If the chi-square is from 6.0 to 9.0, the percentage number is italicized.
* If the chi-square is 9.1 or greater, the percentage number is followed by an asterisk.

was suggested that apart from the different viruses used, some of the differences in the results of the present authors and those of Lichstein et al[2] may have been due to an unobserved hypercalcemia in the animals of the latter workers. The hypercalcemia could have resulted from the diets very low in phosphorus and containing calcium and vitamin D. However, the data presented here show that on diets low in phosphorus and containing vitamin D together with a medium or high amount of calcium (groups 2 and 6), the percentage of deaths was as high or higher than on the complete diet.

The above results can not be due to the inanition resulting from the dietary deficiencies, as it has been demonstrated previously[7,8] that a decrease in food intake to approximately 50% of normal increases the resistance of mice to the Lansing strain of poliomyelitis.

SUMMARY AND CONCLUSIONS

In one series of experiments, the effects of a diet low in phosphorus and vitamin D on the response of mice to the murine-adapted Lansing strain of the virus of poliomyelitis were studied. In a second series of experiments the effects of various dietary levels and combinations of phosphorus, calcium, and vitamin D were investigated. The results of the last experiment of the second series are presented in detail. In the first series it was found that the deficiency did not increase the resistance of the mice to the virus, and in some groups deaths were higher on the deficient than on the complete diets. In the second series this difference, especially during the first several days after inoculation, was still more marked. The increase in susceptibility to the virus was greatest on the more deficient diets. This was particularly true on diets deficient in both phosphorus and calcium. In several cases these differences were statistically significant on most of the days following inoculation.

A FIELD TRIAL OF *SHIGELLA FLEXNERI* III VACCINE. II. SERUM AGGLUTINATION STUDIES

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The problem of shipboard epidemics of shigellosis in the United States Pacific Fleet has been reviewed recently.[1] To summarize, since an extensive outbreak of dysentery due to *Shigella flexneri* III (VIII) aboard ships at anchor in Leyte Gulf, Philippine Islands in the latter part of 1945,[2] this disease has continued to plague various vessels of the Fleet despite strenuous sanitary regulations.[3–5] Almost without exception, the etiologic agent in these outbreaks has been shown to be *Shig. flexneri* III with varying amounts of VII or VIII antigen. It seems probable that the continuing source of infection aboard ships is the asymptomatic carrier, but practical considerations such as the extreme difficulty of detecting all carriers and the inadequacy of present methods for dealing with the carrier state[1,6,7] led to the institution of a trial of vaccination against shigellosis in about 4000 subjects aboard 5 ships of the Fleet which have had recurrent epidemics. A brief outline of the program follows.

After a careful history was taken, each subject was assigned to one of three experimental groups:

Group 1-A: To receive three injectons of parenteral *Shig. flexneri* III vaccine plus 15 placebo tablets.

Group 1-B: To receive three injections of parenteral typhoid vaccine plus 15 tablets of *Shig. flexneri* III vaccine.

Group 2-A: To receive three injections of parenteral typhoid vaccine plus 15 placebo tablets.

The materials employed were:

a. A heat-killed vaccine of *Shig. flexneri* III containing 2.2×10^9 organisms per ml; this was administered in three weekly doses of 0.5, 0.5, and 1.0 ml.

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1. Barnes, L. A., Bennett, I. L., Jr. and Gordon, R. S. 1949, A field trial of Shigella flexneri III vaccine. I. Background, scope, and organization of program. Nav. Med. Bull. **49:** In press.
2. Cheever, F. S. 1946, Dysentery outbreak aboard naval vessels in San Pedro Bay, Philippine Islands. Nav. Med. Bull. **46:** 479–494.
3. Saunders, H. P. 1945, Dysentery epidemic: report of one hundred eighty-four cases. Nav. Med. Bull. **45:** 1209–1211.
4. Williamson, R., Fuller, R., Martin, F. J. and Guchewicz, H. 1948, Bacillary dysentery, Nav. Med. Bull. **48:** 818–821.
5. Mount, R. A. and Floyd, T. M. 1948, A dysentery outbreak aboard a cruiser in Apra Harbor, Guam, Marianas Islands. Nav. Med. Bull. **48:** 240–249.
6. Van Gelder, D. W., Daines, W. P. and Fischer, G. L. 1947, Shigella carriers with special reference to their therapy, including the use of streptomycin. Am. J. Trop. Med. **27:** 225–231.
7. Philbrook, F. R., Barnes, L. A., McCann, W. J., Jr. and Harrison, R. R. 1948, Prolonged laboratory observations on clinical cases and carriers of "Shigella flexneri III" following an epidemic. Nav. Med. Bull. **48:** 405–414.

b. Enteric-coated tablets containing 20×10^9 heat-killed *Shig. flexneri* III organisms; one tablet was given daily for 15 days.

c. Enteric-coated tablets identical in size, shape, and color with the vaccine tablets but containing no organisms.

d. A monovalent typhoid vaccine of the same density and appearance as the parenteral shigella vaccine; the dosage of this material varied aboard each of the ships.

Blood samples were collected on all subjects before vaccination and at intervals thereafter. The results of agglutination studies on the pre- and postinoculation serums are the subject of this report.

REVIEW OF THE LITERATURE

The value of the serum agglutination test as a diagnostic aid or as an indicator of true immunity to shigella infection has long been a subject of controversy. The reasons which may be cited for its inadequacy as a diagnostic test are threefold.

Late rise of agglutinin titer.—Bacillary dysentery in adults is ordinarily a disease of short duration and any rise in agglutinin titer is usually not detectable until after 7 to 14 days[8–13] at a time when most patients have recovered.

The "normal" level of agglutinins.—The problem of what is to be considered a "normal" level of serum agglutinins has been widely discussed.[10] Dudgeon,[11] on the basis of his findings at Salonica during the first World War, held that a titer of 1:50 was specific for Shiga infections and 1:150 for Flexner strains. Ritchie,[14] in 1916, performed tests on 792 serums from subjects with no history of diarrhea and concluded that a titer of 1:64 was diagnostic for Shiga and 1:128 for Flexner infections. He pointed out, however, the necessity for modifying these values upward in special cases. M'Leod and Ritchie[15] concurred with these normal limits as did Gardner,[16] who emphasized the importance of a standard technique for performing agglutinin determinations. Menton[17] and Barbera[18] found agglutinins for shigellae in supposedly normal human serums and postulated that these were the expression of subliminal infection with homologous or associated microorganisms. This view received the support of Widowitz.[19] In 1945

8. Manson-Bahr, P. 1939, The Dysenteric Disorders; the Diagnosis and Treatment of Dysentery, Sprue, Colitis, and Other Diarrheas in General Practice, Baltimore, Williams and Wilkins.
9. Strong, R. P. 1942, Stitt's Diagnosis, Prevention, and Treatment of Tropical Diseases, ed. 6, Philadelphia, Blakiston.
10. Wilson, G. S. and Miles, A. A. 1946, Topley and Wilson's Principles of Bacteriology and Immunity, ed. 3, Baltimore, Williams and Wilkins.
11. Dudgeon, L. S. 1919, Bacillary dysenteries. Brit. M. J. **1**: 448.
12. Davison, W. C. 1920, Bacillary dysentery in children. Bull. Johns Hopkins Hosp. **31**: 225–234.
13. Dawson, D. W. and Moodie, W. 1921, The aetiology of bacillary dysentery in asylums. Lancet, **2**: 225–226.
14. Ritchie, T. R. 1916, On the agglutination reaction of the bacilli of the typhoid-dysentery group with normal sera. Lancet, **1**: 1257–1260.
15. M'Leod, J. W. and Ritchie, A. G. 1920, The serum reaction in bacillary dysentery; observations on agglutination with special reference to the use of freshly prepared bacillary emulsions. J. Path. & Bact. **23**: 217–223.
16. Gardner, A. D. 1921, The normal titer of agglutination for *B. dysenteriae* (Flexner). Lancet, **2**: 1269.
17. Menton, J. 1929, Agglutinins to *B. dysenteriae* in the apparently healthy. Brit. M. J. **1**: 1115–1117.
18. Barbera, G. 1926, The study of agglutinins for the organisms of dysentery. Clin. pediat. **8**: 267.
19. Widowitz, P. 1923, Agglutination of dysentery bacilli in children. Wien. klin. Wchnschr. **36**: 220 (abstracted in J.A.M.A. **80**: 1886).

Klimentova[20] found that the normal titer for Flexner types ranged as high as 1:160. In other studies[21–23] "normal" titers as high as 1:1000 have been found for various types of *Shig. flexneri*. Though early investigators claimed that these normal agglutinins are found predominantly in residents of areas where shigellosis is common, Sears and his coworkers[24] reported in 1938 that the incidence of high titers to several Flexner strains in the northwestern United States (where, according to these authors, shigella infections were rare) differed little from those reported in endemic areas. They concluded that the agglutinating power in serums of normal persons was not related to previous specific infection. Schwabacher et al[25] set normal limits at 1:160 for agglutinins to Flexner types, although they employed a technique for reading tests which gives lower results than those obtained by most workers. Thomas and Levine[26] studied titers of normal serums to several shigellae and concluded that the occurrence of levels exceeding those previously recognized as diagnostic was frequent enough to invalidate results in any individual case.

Failure of proven shigella infections to stimulate specific agglutinins.—Lastly, it has been pointed out that patients with bacteriologically confirmed shigella infections may fail to develop agglutinins or show them in very low titer only. Only about 60% of patients ill with *Shig. sonnei* infections develop specific agglutinins.[10] Ash[27] regarded the agglutination test as "not dependable" in diagnosis, as did Petrie,[28] whose results with the test in asylum outbreaks were inconsistent. Watt,[29] Mackie,[30] and Thomas and Levine[26] in a series of careful studies concluded that the lack of agglutinin response in bacteriologically diagnosed patients is so frequent in occurrence as to render the test almost totally unreliable. Such observations would seem to obviate in large measure the use of serum agglutinin titers to various shigellae as evidence of the relationship between chronic dysentery and chronic ulcerative colitis.[31–33]

20. Klimentova, A. A. 1945, Subcutaneous anavaccine VIEM for dysentery. Am. Rev. Sov. Med. **3**: 135–139.
21. Smolens, J., Halbert, S. P., Mudd, S., Doak, B. W. and Gonzalez, L. M. 1946, Studies with somatic antigen of *Shigella paradysenteriae* (Flexner). J. Immunol. **52**: 41–58.
22. Gardner, A. D. 1923, Agglutination of standard agglutinable strains of Flexner group of dysentery bacilli by non-dysenteric human sera. J. Hyg. **21**: 399–405.
23. Browne, referred to in 9.
24. Sears, H. J., Schwichtenberg, L. and Schwichtenberg, M. 1938, Incidence of agg'utinins for paradysentery bacilli in serums from human beings and from animals. J. Immunol. **35**: 83–92.
25. Schwabacher, H., Ross, M. and Carruthers, H. L. 1943, Agglutination titers of normal sera. Lancet, **1**: 739–741.
26. Thomas, A. R., Jr. and Levine, M. 1946, Serum agglutination reaction in diagnosis of bacillary dysentery. Am. J. Clin. Path. **16**: 98–110.
27. Ash, J. E. 1923, Dysentery with reference particularly to laboratory diagnosis, epidemiology, and treatment. Mil. Surgeon, **52**: 455–509.
28. Petrie, A. A. 1924, The diagnosis of dysenteric infections in mental hospitals, J. Ment. Sc. **70**: 24.
29. Watt, J. and DeCapito, T. M. 1945, Studies of the acute diarrheal diseases XV. The agglutination test in *Shigella paradysenteriae* infections. Pub. Health Rep. **60**: 642–650.
30. Mackie, T. T. 1938, Specificity of agglutinin reaction for *Shigella dysenteriae;* agglutination reaction in chronic bacillary dysentery; serologic and bacteriologic study of 47 cases. Arch. Int. Med. **62**: 783–798.
31. Mackie, T. T. 1934, Ulcerative colitis. I. The relationship between bacillary dysentery and ulcerative colitis. South. M. J. **27**: 492–499.
32. Felsen, J. 1945, Bacillary Dysentery, Colitis and Enteritis, Philadelphia, Saunders.
33. Portis, S. A. 1938, Recurrent diarrhea due to dysentery organisms. J.A.M.A. **110**: 2138–2141.

Source of "natural agglutinins."—There are various observations tending to show that natural antibodies are a by-product in the normal functioning of the antibody-forming apparatus altogether apart from any external stimulus.[10,24,34] However, the balance of the evidence[10,18,35,36] seems to indicate that such agglutinins are the result of subclinical infection with the corresponding organism, contact with an organism having a common antigenic component, or absorption from the intestinal tract of antigenic material from associated bacteria.[37] In the case of shigella infections, the studies of Hardy and his associates[38] have demonstrated the extreme variability of the manifestations of bacillary dysentery, and it requires little urging to believe that the mere inability of any individual to recall on questioning any typical illness does not preclude previous contact with shigellae.

Effect of previous vaccines.—Many attempts have been made to immunize animals and humans against shigellosis. Since Shiga[39] in 1903 advocated the use of a serovaccine which caused the production of demonstrable agglutinins three weeks after inoculation, numerous workers employing heat-killed or irradiated vaccines,[40–47] and modified or detoxified antigens[20,21,48–50] have demonstrated that such inoculation will stimu-

34. Gibson, H. J. 1930, Observations on occurrence, characteristics and specificity of natural agglutinins. J. Hyg. **30**: 337–356.
35. Weil, A. J. 1947, Dysentery; progress report for years 1942 to 1946. J. Immunol. **55**: 363–405.
36. Neter, E. and Gilfillan, R. S. 1939, Agglutinins for *Shigella alkalescens* in sera of normal human individuals and of patients with and without *Shigella alkalescens* infections. Am. J. Hyg. **29**, B: 67–71.
37. Gillespie, H. B., Waugh, M. H. and Serrett, Y. V. 1946, The agglutination of certain types of intestinal bacteria from a healthy human being by the individual's own serum. J. Bact. **52**: 257.
38. Hardy, A. V., Shapiro, R. L., Chant, H. L. and Siegel, M. 1942, Studies of the acute diarrheal diseases IX. *Shigella dysenteriae* infections among institutional inmates. Pub. Health Rep. **57**: 1079–1094.
39. Shiga, K., referred to in 10.
40. Cooper, M. L., Tepper, J. and Keller, H. M. 1944, Active immunization of children with *Shigella paradysenteriae* and *Shigella sonnei*. Proc. Cent. Soc. Clin. Res. **17**: 68.
41. Cooper, M. L., Tepper, J. and Keller, H. M. 1945, Active immunization of children with Shigella vaccines. Proc. Cent. Soc. Clin. Res. **18**: 43–44.
42. Cooper, M. L., Tepper, J. and Keller, H. M. 1948, Studies in dysentery vaccination IV. Primary vaccination in children with monovalent Shigella vaccines. J. Immunol. **60**: 189–204.
43. Felsen, J. and Levenkron, E. 1940, Experimental studies in the production of agglutinins to *B. dysenteriae*. Mil. Surgeon, **87**: 221–229.
44. Felsen, J. and Osofsky, A. G. 1938, The prophylactic use of serums and vaccines in acute bacillary dysentery. J. Infect. Dis. **63**: 298–300.
45. Shaughnessy, H. J., Milzer, A., Neal, J. and Levinson, S. O. 1946, Production of potent inactivated vaccines with ultraviolet irradiation. IV. Vaccination against bacillary dysentery. J. Infect. Dis. **78**: 69–78.
46. Shaughnessy, H. J., Olsson, R. C., Bass, K., Friewer, F. and Levinson, S. O. 1946, Experimental human bacillary dysentery; polyvalent dysentery vaccine in its prevention. J.A.M.A. **132**: 362–368.
47. Hardy, A. V., DeCapito, T. M. and Halbert, S. P. 1948, Studies of the acute diarrheal diseases. XIX. Immunization in shigellosis. Pub. Health Rep. **63**: 685–688.
48. Morgan, W. T. J. and Schutze, H. 1943, Prophylactic inoculation with the O antigen of *Bact. shigae*. Lancet, **2**: 284–285.
49. Perlman, E., Binkley, F. and Goebel, W. F. 1945, Studies on the Flexner group of dysentery bacilli. III. Antibody response in man following the administration of the specific antigen of type V *Shigella dysenteriae* (Flexner). J. Exper. Med. **81**: 349–358.
50. Barnes, F. W., Dewey, M., Henry, S. S. and Lupfer, H. 1947, Chemical detoxification of Flexner dysentery antigen. I. Survey of results. J. Immunol. **56**: 255–261.

late the production of serum agglutinins as well as mouse-protecting antibodies in man and animals. Though it is generally felt that mouse protection furnishes a more reliable index of true immunity than serum agglutinins, in studies with human volunteers[46] and in naturally occurring outbreaks,[47] vaccines which were capable of stimulating the production of both agglutinins and mouse-protecting antibodies had no influence in prevention of the actual disease. Hilliard[51] noted that typhoid vaccine caused a rise in agglutinins for shigellae.

As Weil[35] points out, the lack of correlation between humoral antibody levels and actual immunity in shigellosis may be an indication of the importance of local tissue immunity in this disease, a localized infection of the intestine, rather than a systemic invasion. Whether oral vaccination, despite its poor reputation, offers a hopeful approach remains to be seen. The irregular systemic absorption of antigens from the digestive tract generally leads to a much lower level of humoral antibody production than is seen with parenteral administration. The reader is referred to Dolman's excellent review[52] of this controversial problem.

METHODS

Preinoculation serums were screened at a dilution of 1:160. All serums which fell below this level were counted as 1:80 (the next lower dilution) in computing the preinoculation averages. All serums yielding titers of 1:5120 or greater were retested at least once. Certain of the serums were tested against antigens prepared from organisms other than *Shig. flexneri* III. Such tests were set up in dilutions of 1:20, 1:40, etc., no screening being performed.

To tubes containing serial dilutions of serum an equal volume of antigen was added and the tests were read after standing at room temperature for 18 hours. Parallel observations on 200 serums placed in a waterbath at 55 C for two hours yielded results not significantly different, and the use of the waterbath was abandoned. All tests were read by one of two observers by reflected light against a dark background. The last tube showing clumping of the antigen visible to the unaided eye after shaking was taken as the endpoint.

Antigens.—After repeated subculturing and testing with type-specific serums, smooth colonies were inoculated into brain-heart infusion (Difco) broth and incubated for 8 hours at 37 C. Brain-heart infusion agar slants were inoculated with the broth, and after 18 hours at 37 C, growth was washed from the slants (without scraping) with physiological saline containing 0.25% formalin. The suspension thus obtained was tested for sterility after standing for 48 hours at 4 C and then filtered through 4 layers of gauze. This stock antigen was diluted immediately before using to the point that it transmitted 85% light in the Coleman Jr. spectrophotometer using no. 660 filter.

Adsorption of serums.—Certain serums were retested after adsorption with an excess of antigen in an attempt to delineate certain apparently nonspecific effects of the vaccines employed. To 1.0 ml of packed formalin-killed organisms 1.0 ml of serum was added. This mixture was incubated at 37 C for two hours with stirring every 15 minutes and then allowed to stand for 18 hours at 4 C. After centrifugation, the serum was pipetted off and retested.

RESULTS

Preinoculation serums were divided into three main groups. The first of these, the "normal controls," consisted of serums from subjects who had not been present in any one of the numerous epidemics of shigellosis in the Fleet since 1945 and who gave no history of previous sporadic illness accompanied by abdominal symptoms and fever severe enough to require medical attention. The second, the "exposed controls," consisted of serums of men who, though present in one or more of the epidemics, had never been clinically ill. The last group was made up of serums from men who had been affected in a

51. Hilliard, E. T. 1925, Dysentery in mental hospitals with special reference to the types of organisms involved. M. J. Australia, **2**: 695–698.

52. Dolman, C. E. 1948, Oral immunization. Am. J. M. Sc. **215**: 327–349.

known epidemic, the "ill controls." All subjects were questioned carefully on these points, and in doubtful cases service and health records were consulted. During the history-taking a check-list of all known epidemics in the Fleet was available for reference. The variability in the manifestations of shigellosis, ranging from mild abdominal cramps to an overwhelming and perhaps fatal illness together with the fact that other infections and intoxications may simulate the disease, makes any attempt to determine previous contact with the shigellae by history alone inaccurate. By including in the "exposed" and "ill" groups only serums of men who had been in known epidemics of *Shig. flexneri* III infection since 1945, it was felt that much doubt could be eliminated in these two groups. However, the "normal" group undoubtedly contained some serums from men who had previously been exposed to the genus *Shigella*.

Normal controls.—The average agglutination titer against *Shig. flexneri* III in 1622 serums from subjects with no history of exposure to this organism was 1:321. The first line of table 1 shows the distribution of titers obtained in this group.

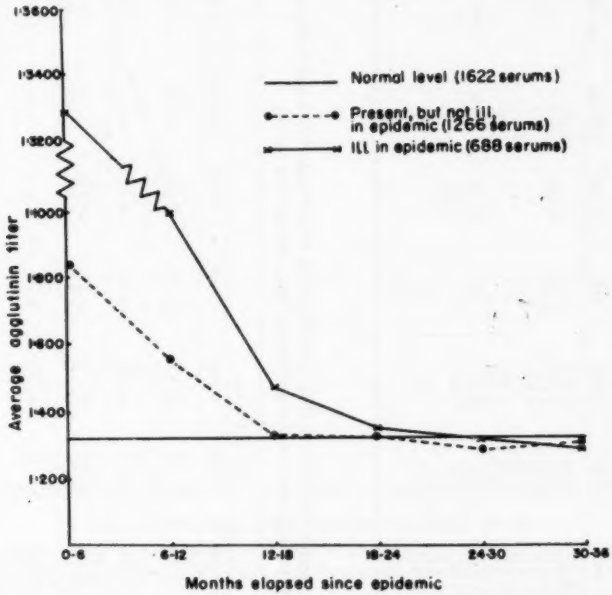

FIG. 1.—Graph showing fall with time of average serum agglutinin titer for *Shig. flexneri* III in subjects known to have been ill or exposed without clinical illness in an epidemic of bacillary dysentary due to this type. (Note that by 18 months in the exposed group and 24 months in the ill group the average titers had fallen to the normal range.)

Exposed controls.—The time elapsed since the exposure of the subjects in this group in epidemics of *Shig. flexneri* III infection varied from less than six months to three years. A total of 1266 serums were tested and the results averaged by six-month intervals from the time of exposure (table 1). The average titers ranged from 1:843 on 290 serums

TABLE 1.—*Percentage distribution of agglutinin titers for* Shig. flexneri *III in 3576 preinoculation serums.*

Group	Serum dilution										Number of serums	Average titer
	<1:160	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:40,960		
Normals	39.7%	17.7%	21.1%	14.4%	5.3%	1.4%	—	—	—	—	1622	1:321
Exposed— 6 mo.	9.6%	21.0%	25.5%	23.8%	11.0%	4.4%	3.4%	0.7%	0.3%	—	290	1:843
Exposed—12 mo.	6.4%	24.1%	28.8%	25.2%	12.0%	2.6%	0.5%	—	—	—	340	1:550
Exposed—18 mo.	37.9%	21.2%	21.6%	11.8%	5.4%	2.0%	—	—	—	—	296	1:330
Exposed—24 mo.	37.0%	22.2%	14.8%	18.5%	7.4%	—	—	—	—	—	108	1:333
Exposed—30 mo.	40.7%	25.9%	16.6%	11.1%	3.7%	1.8%	—	—	—	—	108	1:293
Exposed—36 mo.	45.1%	17.7%	19.4%	11.2%	4.8%	1.6%	—	—	—	—	124	1:301
Ill— 6 mo.	4.2%	20.0%	20.0%	17.1%	11.4%	8.5%	7.1%	5.7%	2.8%	2.8%	70	1:3291
Ill—12 mo.	4.5%	11.2%	21.9%	31.6%	17.3%	10.2%	2.0%	1.0%	—	—	196	1:986
Ill—18 mo.	31.7%	15.9%	27.0%	12.8%	10.1%	2.0%	—	0.6%	—	—	148	1:469
Ill—24 mo.	20.0%	40.0%	14.4%	20.0%	6.6%	—	—	—	—	—	90	1:336
Ill—30 mo.	42.8%	7.1%	32.1%	7.1%	10.7%	—	—	—	—	—	112	1:320
Ill—36 mo.	48.6%	19.4%	11.1%	6.9%	11.1%	—	—	—	—	—	72	1:296

Note that by 18 months in the exposed group and 24 months in the ill group the average titers had dropped to the normal range, remaining at this level thereafter.

TABLE 2.—*Percentage distribution by ship of agglutinin titers for* Shig. flexneri *III in 809 preinoculation and 1193 postinoculation serums from subjects receiving three weekly injections of* Shig. flexneri *III vaccine.*

Ship	Serum	Serum dilution										Number of serums	Average titer
		<1:160	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:40,960		
1	Preinoculation	49.1%	14.9%	25.1%	7.7%	2.9%	—	—	—	—	—	167	1:231
	20–21 days	—	—	4.2%	8.5%	20.6%	30.6%	15.0%	9.2%	9.2%	2.1%	140	1:5618
	75–77 days	—	5.2%	11.1%	17.7%	29.6%	21.7%	10.5%	3.9%	—	—	152	1:2035
2	Preinoculation	10.6%	20.4%	30.2%	28.6%	6.5%	3.2%	—	—	—	—	122	1:489
	1–2 days	—	2.6%	4.6%	12.5%	20.3%	28.9%	14.4%	9.2%	5.9%	1.3%	152	1:4536
3	Preinoculation	31.5%	28.0%	16.3%	15.7%	6.4%	1.7%	—	—	—	—	171	1:348
	16–17 days	—	0.7%	3.6%	10.1%	19.5%	28.2%	17.3%	10.8%	5.7%	3.6%	138	1:5726
	55–56 days	0.9%	2.8%	13.0%	27.1%	32.7%	12.1%	1.8%	3.7%	3.7%	1.8%	107	1:2960
4	Preinoculation	76.0%	10.8%	10.3%	2.1%	0.5%	—	—	—	—	—	184	1:132
	15–16 days	1.5%	4.7%	22.9%	19.0%	29.7%	10.7%	5.3%	3.0%	1.5%	1.5%	131	1:2382
	100–101 days	1.8%	16.8%	42.0%	26.1%	9.3%	3.7%	—	—	—	—	107	1:545
5	Preinoculation	11.5%	21.8%	24.2%	27.2%	13.3%	1.8%	—	—	—	—	165	1:513
	30–31 days	—	7.4%	6.6%	20.0%	17.0%	14.8%	10.3%	8.1%	8.8%	6.6%	135	1:6674
	46–47 days	—	1.5%	7.6%	32.0%	25.9%	11.4%	5.3%	4.7%	6.1%	5.3%	131	1:5039

at six months to 1:301 on 124 serums at three years. By 18 months the average titer had returned to near the "normal" level and thereafter remained in this range (fig. 1).

Ill controls.—A total of 688 serums fell into this group. The average titer for 70 serums from subjects known to have been ill with *Shig. flexneri* III infections during the previous six months was 1:3291. Average titers at six month intervals fell steadily, reaching the "normal" range by two years and leveling off. Table 1 shows the distribution of titers in this group. Figure 1 illustrates the steady fall of agglutinins with time.

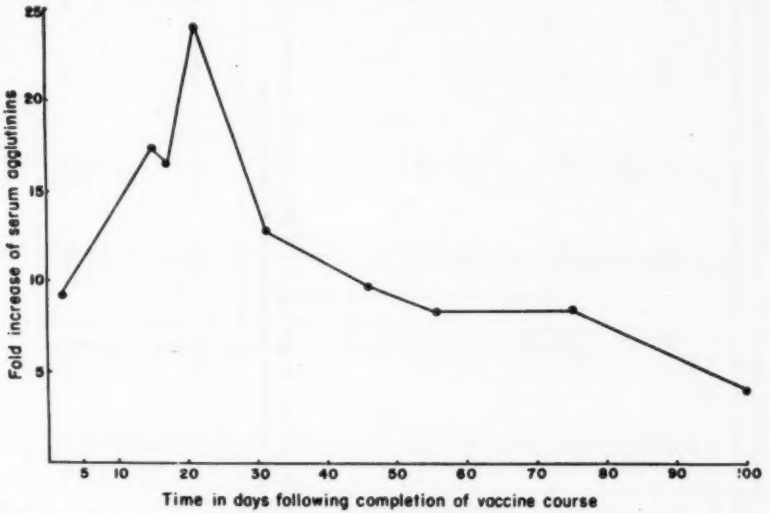

FIG. 2.—Graph of rise and fall of agglutinating antibody titer for *Shig. flexneri* III (Be) in 1193 serums from subjects vaccinated parenterally with the homologous strain.

Effect of parenteral Shig. flexneri *III vaccine.*—A total of 1193 serums collected at intervals of 2 to 101 days following the third dose of parenteral vaccine was available for study. The serums were collected from different ships and the average preinoculation titers for the "1-A" groups aboard the various ships in the study varied so widely (1:132 to 1:513), depending upon the length of time since the last epidemic aboard each ship, that a statement of the results on the basis of average titers would be misleading. Therefore, the postinoculation titers were compared to preinoculation levels for the individual ships and the fold increase of agglutinins was computed. Figure 2 traces the rise and fall of agglutinating antibody following parenteral vaccination. The peak (24.2-fold) came 21 days following the last injection of vaccine. Table 2 shows the distribution of the titers obtained for each ship.

Effect of oral Shig. flexneri *III vaccine.*—A total of 1124 serums collected at intervals following completion of oral vaccination in the "1-B" group were tested. As was the case with the "1-A's," the ship to ship variation in preinoculation averages (1:156 to 1:478) made the fold increase method of comparing results preferable. Figure 3 shows the rise

TABLE 3.—*Percentage distribution by ship of agglutinin titers for* Shig. flexneri *III in 724 preinoculation and 1124 postinoculation serums from subjects receiving 15 tablets of* Shig. flexneri *III vaccine plus varying amounts of typhoid vaccine.*

Ship	Serum	Serum dilution										Number of serums	Average titer
		<1:160	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:40,960		
1	Preinoculation	45.4%	18.4%	23.3%	11.0%	1.2%	—	—	0.7%	—	—	163	1:272
	20–21 days	3.0%	6.7%	25.5%	30.0%	19.5%	13.5%	—	0.7%	—	0.7%	13	1:1269
	75–76 days	7.5%	8.2%	22.5%	25.5%	26.3%	8.2%	0.7%	0.7%	—	—	133	1:919
3	Preinoculation	16.1%	29.5%	30.1%	14.1%	7.3%	2.0%	0.6%	—	—	—	149	1:420
	16–17 days	1.5%	1.2%	15.7%	32.2%	30.0%	12.0%	3.7%	2.2%	0.7%	—	133	1:1531
	55–56 days	0.9%	6.4%	17.4%	38.4%	27.4%	2.7%	1.8%	1.8%	2.7%	—	109	1:1581
4	Preinoculation	65.6%	17.1%	14.1%	2.4%	—	0.6%	—	—	—	—	163	1:156
	15–16 days	8.1%	9.6%	45.9%	22.9%	11.1%	1.5%	—	—	0.7%	—	135	1:647
	100–101 days	2.8%	31.4%	40.9%	21.9%	1.9%	0.9%	—	—	—	—	105	1:372
5	Preinoculation	8.9%	23.7%	31.4%	26.2%	8.3%	1.2%	—	—	—	—	156	1:453
	30–31 days	2.7%	13.5%	26.1%	35.1%	9.9%	0.9%	3.6%	4.5%	2.7%	0.8%	111	1:2419
	46–47 days	—	17.9%	29.9%	24.7%	5.9%	10.2%	6.8%	2.5%	0.8%	0.8%	117	1:1760
2*	Preinoculation	3.2%	26.8%	32.2%	27.9%	9.6%	1.0%	—	—	—	—	93	1:478
	1–2 days	2.0%	8.1%	10.8%	32.4%	23.6%	18.9%	2.0%	2.0%	—	—	148	1:1355

* Subjects on this ship received oral *Shig. flexneri* III vaccine only, without parenteral typhoid placebo.

TABLE 4.—*Percentage distribution by ship of agglutinin titers for* Shig. flexneri *III in 942 preinoculation and 1108 postinoculation serums from subjects receiving oral placebo tablets with varying amounts of typhoid vaccine.*

Ship	Serum	Serum dilution										Number of serums	Average titer
		<1:160	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	1:20,480	1:40,960		
1*	Preinoculation	49.1%	19.0%	20.2%	8.0%	3.4%	—	—	—	—	—	346	1:230
	20–21 days	20.3%	22.8%	26.8%	20.3%	8.4%	1.4%	—	—	—	—	285	1:411
2†	Preinoculation	11.0%	24.8%	30.1%	22.5%	8.7%	2.6%	—	—	—	—	262	1:470
	1–2 days	10.6%	28.8%	31.3%	18.5%	8.5%	2.1%	—	—	—	—	329	1:437
3‡	Preinoculation	4.7%	17.6%	19.4%	29.6%	17.0%	9.2%	1.4%	0.6%	—	—	334	1:877
	16–17 days	0.7%	4.6%	16.4%	28.6%	26.5%	18.6%	3.9%	0.4%	—	—	279	1:1299
	55–56 days	3.2%	13.9%	26.0%	26.0%	17.2%	5.1%	4.1%	1.4%	1.8%	0.9%	215	1:7145

* Received oral placebo and three weekly doses of 0.5, 0.5, and 1.0 ml typhoid vaccine.
† Received oral placebo only.
‡ Received oral placebo and three weekly doses of 0.3, 0.3, and 0.3 ml typhoid vaccine.

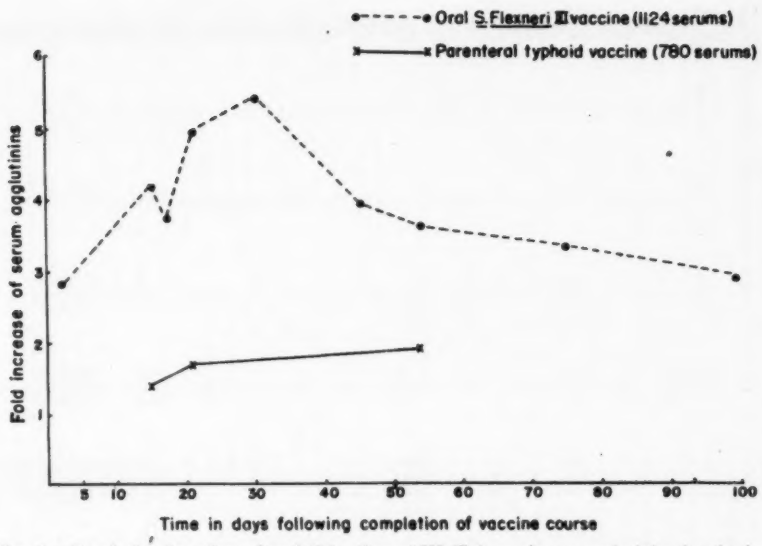

FIG. 3.—Graph showing effect of oral *Shig. flexneri* III (Be) vaccine on agglutinin titer for homologous organism as compared with the effect of parenteral typhoid vaccine.

TABLE 5.—*Percentage distribution of agglutinin titers for various shigella types in 100 serums from subjects giving no history of diarrheal illness or contact with epidemic shigellosis.*

Antigen	Serum dilution						
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
Shig. flexneri I	42%	32%	11%	11%	3%	1%	—
Shig. flexneri II	35%	27%	16%	13%	6%	3%	—
Shig. flexneri III	7%	35%	38%	14%	6%	2%	—
Shig. flexneri IV	41%	29%	14%	10%	4%	2%	—
Shig. flexneri V	48%	34%	7%	7%	4%	—	—
Shig. flexneri VI	41%	26%	17%	14%	2%	—	—
Shig. flexneri VII	13%	24%	29%	18%	11%	5%	—
Shig. flexneri VIII	14%	33%	40%	5%	4%	2%	2%
Shig. flexneri IX	59%	19%	15%	7%	2%	—	—
Shig. flexneri X	61%	17%	7%	6%	5%	3%	1%
Shig. flexneri XI	71%	23%	3%	3%	—	—	—
Shig. flexneri XII	52%	23%	12%	8%	5%	—	—
Shig. flexneri XIII	25%	37%	21%	14%	3%	1%	—
Shig. flexneri XIV	61%	28%	7%	4%	—	—	—
Shig. flexneri III (Be)*	14%	30%	21%	17%	18%	5%	3%
Shig. flexneri III (VII)†	14%	23%	23%	17%	13%	9%	1%
Shig. flexneri III (VIII)†	35%	27%	28%	10%	1%	1%	—
Shig. flexneri III (VIII)†	24%	25%	30%	19%	2%	—	—
Shig. dispar I	62%	20%	7%	7%	3%	1%	—
Shig. dispar II	73%	13%	8%	6%	—	—	—
Shig. dispar III	62%	27%	6%	3%	2%	—	—
Shig. dispar IV	64%	14%	9%	7%	5%	1%	—
Shig. Sachs Q454	93%	6%	1%	—	—	—	—
Shig. Sachs Q771	93%	7%	—	—	—	—	—
Shig. Sachs Q902	90%	10%	—	—	—	—	—
Shig. Sachs Q1030	94%	6%	—	—	—	—	—
Shig. Sachs Q1167	96%	3%	1%	—	—	—	—
Shig. alkalescens	48%	34%	6%	6%	4%	2%	—
Shig. ambigua	64%	23%	10%	3%	—	—	—
Shig. dysenteriae	87%	7%	6%	—	—	—	—
Shig. rabaulensis	37%	28%	16%	11%	5%	3%	—
Shig. sonnei	41%	33%	14%	8%	4%	—	—
S. typhi	31%	18%	21%	23%	7%	—	—

* The strain employed in the vaccine.
† Strains isolated from epidemics in the Pacific Fleet.

TABLE 6.—*Percentage distribution of agglutinin titers for various shigella types in preinoculation and postinoculation serums.*

Antigen	Serum	Serum dilution										Number of serums
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10,240	
Shig. flexneri I	Preinoculation	23.9%	42.4%	23.0%	9.2%	0.9%	—	0.9%	—	—	—	107
	1–30 days	10.1%	39.5%	28.5%	12.8%	4.6%	—	0.9%	—	—	—	107
	30–100 days	14.3%	41.8%	35.2%	7.7%	—	—	—	—	—	—	90
Shig. flexneri II	Preinoculation	18.4%	32.2%	30.3%	14.7%	2.7%	—	—	—	—	—	107
	1–30 days	4.6%	33.1%	32.1%	19.3%	6.4%	—	0.9%	—	—	—	107
	30–100 days	7.7%	40.7%	42.9%	11.0%	2.2%	—	—	—	—	—	90
Shig. flexneri III	Preinoculation	11.0%	31.2%	29.4%	23.0%	2.7%	—	—	—	—	—	107
	1–30 days	0.9%	11.0%	31.2%	28.5%	17.5%	3.7%	2.7%	0.9%	—	—	107
	30–100 days	3.3%	22.0%	28.6%	28.6%	11.0%	3.3%	1.1%	1.1%	—	—	90
Shig. flexneri IV	Preinoculation	43.3%	36.8%	16.5%	1.8%	—	—	—	—	—	—	107
	1–30 days	23.0%	38.6%	30.3%	3.7%	0.9%	—	—	—	—	—	107
	30–100 days	30.8%	53.9%	12.1%	2.2%	—	—	—	—	—	—	90
Shig. flexneri V	Preinoculation	37.7%	46.0%	12.8%	2.7%	—	—	—	—	—	—	107
	1–30 days	24.9%	26.0%	22.0%	6.4%	0.9%	0.9%	—	—	—	—	107
	30–100 days	33.0%	39.6%	24.2%	2.2%	—	—	—	—	—	—	90
Shig. flexneri VI	Preinoculation	61.6%	33.1%	2.7%	—	0.9%	—	—	—	—	—	107
	1–30 days	34.9%	46.0%	13.8%	1.8%	—	—	—	—	—	—	107
	30–100 days	41.8%	42.9%	14.3%	—	—	—	—	—	—	—	90
Shig. flexneri VII	Preinoculation	11.0%	13.8%	33.1%	26.7%	12.8%	2.7%	—	—	—	—	107
	1–30 days	1.8%	5.5%	18.4%	29.4%	22.0%	15.6%	3.7%	—	—	—	107
	30–100 days	4.4%	12.1%	5.4%	41.8%	17.6%	5.5%	2.2%	—	—	—	90
Shig. flexneri VIII	Preinoculation	17.4%	31.2%	39.5%	7.3%	2.7%	—	—	—	—	—	107
	1–30 days	7.4%	23.0%	34.0%	23.9%	8.3%	1.8%	—	—	—	—	107
	30–100 days	7.7%	28.6%	40.7%	14.3%	5.5%	2.2%	—	—	—	—	90
Shig. flexneri IX	Preinoculation	69.0%	23.9%	4.6%	0.9%	0.9%	—	—	—	—	—	107
	1–30 days	44.2%	34.0%	17.5%	0.9%	1.8%	—	—	—	—	—	107
	30–100 days	52.8%	36.3%	9.9%	—	—	—	—	—	—	—	90
Shig. flexneri X	Preinoculation	60.7%	24.8%	11.9%	0.9%	—	—	—	—	—	—	107
	1–30 days	46.0%	27.6%	18.4%	4.6%	—	—	—	—	—	—	107
	30–100 days	63.8%	24.2%	8.8%	2.2%	—	—	—	—	—	—	90
Shig. flexneri XI	Preinoculation	53.3%	34.9%	5.5%	1.8%	—	—	—	—	—	—	107
	1–30 days	45.1%	32.1%	15.6%	2.7%	—	—	—	—	—	—	107
	30–100 days	46.2%	42.9%	13.2%	2.2%	—	—	—	—	—	—	90
Shig. flexneri XII	Preinoculation	44.2%	46.0%	9.2%	0.9%	—	—	—	—	—	—	107
	1–30 days	42.4%	30.3%	19.3%	3.7%	0.9%	0.9%	—	—	—	—	107
	30–100 days	44.0%	40.7%	13.2%	2.2%	—	—	—	—	—	—	90
Shig. flexneri XIII	Preinoculation	8.3%	35.8%	19.3%	15.6%	14.7%	3.7%	0.9%	—	—	—	107
	1–30 days	1.8%	14.7%	19.3%	23.0%	20.2%	11.9%	3.7%	1.8%	—	—	107
	30–100 days	7.7%	14.3%	29.7%	24.2%	15.4%	6.6%	—	—	—	—	90
Shig. flexneri XIV	Preinoculation	45.1%	33.1%	16.5%	2.7%	1.8%	—	—	—	—	—	107
	1–30 days	39.5%	33.1%	16.5%	1.8%	4.6%	0.9%	—	—	—	—	107
	30–100 days	46.2%	34.1%	14.3%	—	2.2%	1.1%	1.1%	—	—	—	90
Shig. flexneri III (VII)	Preinoculation	2.7%	14.7%	19.3%	24.9%	19.3%	12.8%	2.7%	1.8%	—	—	107
	1–30 days	0.9%	9.2%	11.9%	23.0%	13.8%	24.9%	7.3%	3.7%	0.9%	0.9%	107
	30–100 days	1.1%	11.0%	16.5%	26.4%	22.0%	14.3%	6.6%	1.1%	—	1.1%	90
Shig. flexneri III (VIII)	Preinoculation	12.8%	28.5%	28.5%	22.0%	4.6%	1.8%	—	—	—	—	107
	1–30 days	10.1%	16.6%	23.0%	26.7%	19.3%	5.5%	—	—	—	—	107
	30–100 days	9.9%	29.7%	30.8%	18.7%	20.9%	2.2%	—	—	—	—	90

TABLE 6.—*Continued.*

Shig. flexneri III (VIII)	Preinoculation	12.8%	26.7%	23.9%	24.9%	8.3%	1.8%	—	—	—	—	107
	1–30 days	5.5%	10.1%	19.3%	24.0%	19.3%	12.8%	4.6%	—	0.9%	—	107
	30–100 days	8.8%	13.2%	24.2%	31.9%	20.9%	3.3%	1.1%	1.1%	—	—	90
Shig. flexneri III (Be)	Preinoculation	3.7%	6.4%	16.5%	24.9%	27.6%	11.9%	5.5%	0.9%	—	—	107
	1–30 days	1.8%	2.7%	3.7%	6.4%	29.4%	18.4%	13.8%	10.1%	4.6%	1.8%	107
	30–100 days	1.1%	6.6%	7.7%	20.9%	23.1%	17.6%	16.5%	3.3%	3.3%	—	90
Shig. dispar I	Preinoculation	14.7%	24.9%	27.6%	22.0%	7.3%	0.9%	—	—	—	—	107
	1–30 days	23.9%	22.1%	9.2%	11.9%	13.8%	11.0%	—	—	—	—	107
	30–100 days	39.6%	24.2%	17.6%	5.5%	8.8%	2.2%	1.1%	—	—	—	90
Shig. dispar II	Preinoculation	63.4%	14.7%	13.7%	5.5%	0.9%	—	—	—	—	—	107
	1–30 days	52.4%	16.6%	14.7%	9.2%	3.7%	—	—	—	—	—	107
	30–100 days	69.3%	17.6%	12.1%	—	—	—	—	—	—	—	90
Shig. dispar III	Preinoculation	41.5%	34.0%	11.9%	5.5%	6.4%	—	—	—	—	—	107
	1–30 days	29.4%	28.5%	22.0%	11.0%	5.5%	—	0.9%	—	—	—	107
	30–100 days	45.1%	31.9%	18.7%	3.3%	—	—	—	—	—	—	90
Shig. dispar IV	Preinoculation	57.0%	30.3%	8.3%	1.8%	0.9%	—	—	—	—	—	107
	1–30 days	56.1%	24.9%	12.8%	1.8%	—	0.9%	—	—	—	—	107
	30–100 days	66.0%	26.4%	6.6%	—	—	—	—	—	—	—	90
Shig. Sachs Q454	Preinoculation	77.3%	15.6%	5.5%	—	—	—	—	—	—	—	107
	1–30 days	75.5%	16.5%	4.6%	—	—	—	—	—	—	—	107
	30–100 days	78.1%	17.6%	3.3%	—	—	—	—	—	—	—	90
Shig. Sachs Q771	Preinoculation	84.6%	12.8%	0.9%	—	—	—	—	—	—	—	107
	1–30 days	76.4%	15.6%	4.6%	—	—	—	—	—	—	—	107
	30–100 days	84.7%	14.3%	—	—	—	—	—	—	—	—	90
Shig. Sachs Q902	Preinoculation	81.9%	12.8%	3.7%	—	—	—	—	—	—	—	107
	1–30 days	73.7%	18.8%	6.4%	—	—	—	—	—	—	—	107
	30–100 days	75.9%	18.7%	4.4%	—	—	—	—	—	—	—	90
Shig. Sachs Q1030	Preinoculation	86.4%	10.1%	0.9%	—	—	—	—	—	—	—	107
	1–30 days	76.4%	15.6%	2.7%	1.8%	—	—	—	—	—	—	107
	30–100 days	83.6%	13.2%	2.2%	—	—	—	—	—	—	—	90
Shig. Sachs Q1167	Preinoculation	84.6%	10.1%	1.8%	—	1.8%	—	—	—	—	—	107
	1–30 days	78.2%	13.8%	2.7%	1.8%	—	—	—	—	—	—	107
	30–100 days	88.0%	8.8%	2.2%	—	—	—	—	—	—	—	90
Shig. alkalescens	Preinoculation	29.4%	43.3%	15.6%	—	7.3%	3.7%	—	—	—	—	107
	1–30 days	20.2%	34.0%	34.9%	—	0.9%	—	—	—	—	—	107
	30–100 days	29.7%	41.8%	24.2%	3.3%	—	—	—	—	—	—	90
Shig. ambigua	Preinoculation	39.5%	36.8%	17.5%	4.6%	—	—	—	—	—	—	107
	1–30 days	30.3%	35.0%	21.1%	9.2%	0.9%	—	—	—	—	—	107
	30–100 days	46.2%	36.3%	12.1%	3.3%	1.1%	—	—	—	—	—	90
Shig. dysenteriae	Preinoculation	75.4%	16.5%	4.6%	1.8%	—	—	—	—	—	—	107
	1–30 days	69.0%	19.3%	8.3%	—	—	—	—	—	—	—	107
	30–100 days	77.0%	16.5%	5.5%	—	—	—	—	—	—	—	90
Shig. rabaulensis	Preinoculation	20.2%	34.9%	18.4%	17.5%	6.4%	0.9%	—	—	—	—	107
	1–30 days	15.6%	12.8%	20.2%	21.1%	21.1%	5.5%	—	—	—	—	107
	30–100 days	19.8%	22.0%	19.8%	23.1%	14.3%	—	—	—	—	—	90
Shig. sonnei	Preinoculation	44.1%	37.7%	9.2%	1.8%	—	0.9%	—	—	—	—	107
	1–30 days	45.1%	31.2%	17.5%	0.9%	1.8%	0.9%	—	—	—	—	107
	30–100 days	46.2%	41.8%	11.0%	—	—	—	—	—	—	—	90
S. typhi	Preinoculation	27.6%	38.6%	19.3%	11.9%	—	0.9%	—	—	—	—	107
	1–30 days	17.4%	22.0%	26.7%	20.2%	7.4%	2.7%	—	—	—	—	107
	30–100 days	23.1%	30.8%	30.8%	14.3%	1.1%	—	—	—	—	—	90

Note that titers for *Shig. flexneri* III, VII, and XIII, as well as *Shig. rabaulensis* showed consistent rises after vaccination. Less regular rises were also noted for *Shig. flexnerii* VIII, *Shig. dispar* I and III, and *S. typhi*.

and fall of agglutinins in this group. Though the oral vaccine resulted in a definite increase in agglutinin titers, the magnitude of the changes did not approach that following parenteral immunization. The peak of the rise (5.3-fold) was reached at 30 to 31 days, about 10 days later than the maximum in the parenterally inoculated group. Table 3 gives the distribution of the titers in this group by ship. The total dose of oral vaccine was 300×10^9 organisms during a 15-day period as compared with 4.4×10^9 subcutaneously in the "1-A's."

On 4 of the 5 ships in the study, the subjects receiving oral vaccine were also given parenteral typhoid vaccine as a placebo. In order to assess the effect of this material upon the agglutination test for *Shig. flexneri* III, 780 serums collected from the "2-A" experimental group (which received typhoid vaccine subcutaneously plus an oral placebo) were tested. A slight but definite rise was noted as can be seen in figure 3. Table 4 shows the distribution of titers in these subjects.

It should be noted that a portion of the rise in the "1-B" group was apparently due to the parenteral placebo. In this connection, however, 148 serums from ship no. 2, collected two days after completion of the course of oral vaccine, were from subjects who had received no parenteral typhoid vaccine and gave an average titer of 1:1355, an increase of 2.8-fold over the preinoculation average of 1:478.

Preinoculation averages from 262 subjects in the "2-A" group on ship no. 2 yielded an average titer of 1:470. Two days after a course of oral placebo tablets unaccompanied by typhoid vaccine had been completed, 329 serums from this group showed no significant change, averaging 1:437 (table 4).

Agglutinin titers of normal serums for various shigella types.—One hundred serums from the "normal" group were tested with 33 antigens prepared from shigella types; a typhoid "O" strain was included for general interest. Most of the strains for these tests were obtained from the culture collection of the Naval Medical Research Institute at Bethesda. Table 5 shows the distribution of the titers obtained with these serums.

Effect of Shig. flexneri *III vaccine upon agglutinin titers for other shigella types.*—Table 6 presents the results obtained with these various antigens in pre- and postinoculation serums. While the rise in titer for the various strains of *Shig. flexneri* III predominated, titers to certain other types rose quite regularly, namely *Shig. flexneri* VII, *Shig. flexneri* XIII, and *Shig. rabaulensis*. Less consistent rises were also noted for *Shig. flexneri* VIII, *Shig. dispar* I, *Shig. dispar* III, and *Salmonella typhi*.

For three strains isolated from epidemics in the Pacific Fleet, a *Shig. flexneri* III (VII) and two *Shig. flexneri* III (VIII) strains, titers after vaccination were usually lower than those for the homologous strain, *Shig. flexneri* III (Be), in the vaccine.

Effect of adsorption of serums upon agglutinin titer for other shigella types.—Thirty pairs of serums consisting of one pre- and one postinoculation specimen were chosen for adsorption studies. The antigens used for adsorption were *Shig. flexneri* III (Be), *Shig. flexneri* VII, *Shig. flexneri* XIII, *Shig. dispar* I, and *Shig. rabaulensis*.

Serums adsorbed with *Shig. flexneri* III showed a significant reduction in titer for all but *Shig. flexneri* XIII. Adsorption with *Shig. flexneri* VII reduced titers for the other types although the effect on levels for *Shig. flexneri* XIII was inconsistent. Serums adsorbed with *Shig. flexneri* XIII showed reduced

titers for *Shig. flexneri* III, but levels for the other types remained unaffected. *Shig. dispar* I was effective in reducing titers for all types but *Shig. rabaulensis*. *Shig. rabaulensis* lowered titers for *Shig. flexneri* III and VII but had no effect on *Shig. flexneri* XIII and *Shig. dispar* I.

Table 7 outlines the results of these studies.

Normal level of agglutinins for shigellae.—It is apparent from the data presented above that the serums of many "normal" subjects contain levels of agglutinins for various types of *Shig. flexneri* which exceed the traditionally accepted normal range of about 1:150. The explanation of the source of these antibodies is not possible from the data in this study. The demonstration that a vaccine of *Shig. flexneri* III caused significant rises in antibody for numerous other shigella types would seem, however, to lend support to the theory that such natural agglutinins may have their origin in subliminal infection with the same or antigenically related microorganisms. Apparently the influence of exposure to a shigella, with or without clinical illness, upon the level of serum agglutinins, is negligible after two years. This applies only to the "resting level" of antibody. The implications of such previous exposure in terms of confusing anamnestic reactions are obvious and probably of much longer duration. Because of overlapping antigenic composition, even the demonstration of a rising titer to a specific strain of shigella in the serum of an ill subject probably furnishes no reliable guide to the species or even the genus of the infecting organism and is certainly no substitute for bacteriological studies.

TABLE 7.—*Effect of adsorption of serums upon titers for various shigella types which rose most often following administration of vaccine.*

Reacting antigen	Adsorbing antigen				
	Shig. flexneri III	*Shig. flexneri* VII	*Shig. flexneri* XIII	*Shig. dispar* I	*Shig. rabaulensis*
Shig. flexneri III	+	+	+	+	+
Shig. flexneri VII	+	+	−	±	+
Shig. flexneri XIII	−	±	+	+	−
Shig. dispar I	+	+	−	+	−
Shig. rabaulensis	+	+	−	−	+

Note that while adsorption with *Shig. flexneri* III or *Shig. dispar* I reduced agglutinin titer for *Shig. flexneri* XIII, there was no reduction in titer for these two types following adsorption with *Shig. flexneri* XIII.
+ indicates that the adsorbing antigen reduced the titer for the reacting antigen.
− indicates that the adsorbing antigen did not affect the titer for the reacting antigen.
± indicates variable results.

DISCUSSION

Effect of vaccines on serum agglutinins.—The vaccine administered parenterally in this study caused the production of serum agglutinins for the specific shigella type in the vaccine and to a lesser extent for several other types, probably a reflection of the overlapping of minor antigenic components in this group. The rise of agglutinins for shigellae after typhoid vaccine in control subjects strengthens this view. This production of agglutinating antibody agrees substantially with the experience of others with similar vaccines. The question of the significance of levels of agglutinins in terms of human immunity to shigellosis is not touched upon in this study. The lower level of response to the oral administration of vaccine was not unexpected in view of the observations of other investigators.

The peak of agglutinin production after oral immunization came at 30 to 31 days, somewhat later than that following parenteral vaccination, which came at 20 to 21 days.

SUMMARY AND CONCLUSIONS

1. Agglutination tests were per-

formed upon serums obtained from 3576 human subjects before and at varying intervals after the completion of a course of parenteral or oral *Shigella flexneri* III vaccination.

2. The average titer for *Shig. flexneri* III of 1622 serums from subjects with no history of previous exposure to this organism was 1:321.

3. Average titers of 1266 serums from individuals exposed, but not clinically ill, in shipboard epidemics of dysentery fell from 1:843 at 6 months following exposure to 1:330 at 18 months and remained within the "normal range" thereafter.

4. Tests on 688 serums from subjects who were clinically ill with *Shig. flexneri* III infections showed an average titer of 1:3291 at 6 months after illness, dropping to 1:336 at two years and leveling off thereafter.

5. The peak of agglutinin production after completion of a course of three weekly injections of parenteral *Shig. flexneri* III vaccine was 24.2-fold at 20 to 21 days.

6. The peak after oral vaccination (one tablet daily for 15 days) was 5.3-fold at 30 to 31 days.

7. The vaccines employed stimulated the production of agglutinins for *Shig. flexneri* VII and XIII and *Shig. rabaulensis*, with less regular responses for *Shig. flexneri* VIII, *Shig. dispar* I and III, and *S. typhi*.

8. Adsorption studies appeared to support the hypothesis that "natural antibodies" for shigellae are the result of subliminal infection with homologous or antigenically related organisms.

9. It was concluded that the vaccines administered were capable of stimulating the production of serum agglutinins for homologous and related types and that the antigenic relationships of the shigellae render the serum agglutination test unreliable in diagnosis.

10. The problem of the relationship of humoral antibody levels to actual human immunity to shigellosis is not dealt with in this study.

INHIBITION OF PYRUVATE OXIDATION IN THE MALARIAL PARASITE *PLASMODIUM GALLINACEUM* BY QUININE TREATMENT OF THE HOST

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It was shown in a recent publication[1] that the intravenous administration of 20 mg quinine per kg body weight to chickens infected with *Plasmodium gallinaceum* causes marked changes in the metabolism of parasitized erythrocytes taken from the infected chickens 24 hours after the quinine injection. The complete oxidation of glucose to carbon dioxide and water is inhibited by quinine, while the anaerobic breakdown of glucose to lactate and pyruvate is unaffected. This finding suggests that quinine affects the carbohydrate metabolism of *P. gallinaceum* by inhibiting the oxidation of pyruvate. A similar conclusion had previously been reached by Silverman et al[2] after study of the inhibitions produced by the in vitro addition of quinine to chicken erythrocytes parasitized with *P. gallinaceum*.

The present report describes the effect of quinine treatment of chickens infected with *P. gallinaceum* upon the metabolism of erythrocyte-free parasites, in which the intermediate steps in the oxidation of carbohydrate may be studied conveniently.[3] Free parasites from quinine-treated chickens convert glucose to lactate at a normal rate, but they oxidize pyruvate at less than half the rate of free parasites from untreated chickens. Pyruvate oxidation in quinine-treated free parasites appears to be inhibited at the first step in its oxidative removal, presumably the formation of a reactive two-carbon particle.

MATERIALS AND METHODS

Crossbred chickens of 1.5 to 2.0 kg weight were infected with *P. gallinaceum** by the intravenous injection of 0.5 to 3.0 ml of heavily parasitized blood. When the infection had progressed to the desired stage, 25 to 50 ml blood were withdrawn from the heart of each chicken into one-tenth volume of 10% sodium citrate as an anticoagulant. Suspensions of intact, parasitized erythrocytes were prepared as previously described.[1] Erythrocyte-free parasites were obtained by hemolyzing parasitized erythrocytes with rabbit chicken erythrocyte hemolysin and guinea pig complement according to the method given in detail by Speck et al.[3]

Quinine dihydrochloride, Merck U. S. P., was dissolved in sterile distilled water just before use and slowly injected into the wing veins of the parasitized chickens.

Parasite number, parasite distribution, and parasite surface area in parasitized erythrocyte suspensions were determined by examination of Giemsa-stained blood smears as in previous investigations.[1,2] The parasite surface area of a

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1. Moulder, J. W. 1948, J. Infect. Dis. **83**: 262–270.

2. Silverman, M., Ceithaml, J., Taliaferro, L. G. and Evans, E. A., Jr. 1944, J. Infect. Dis. **75**: 212–230.

3. Speck, J. F., Moulder, J. W. and Evans, E. A., Jr. 1946, J. Biol. Chem. **164**: 119–114.

* Strain 8A as designated by the Committee on Terminology of Strains of Avian Malaria of the American Society of Parasitologists.

free parasite preparation was assumed to be that of the parasitized erythrocyte suspension from which it was derived. Reticulocyte counts were made on blood smears stained with brilliant cresyl blue.

Glucose was determined by the method of Nelson,[4] and pyruvate was measured by the method of Friedemann and Haugen.[5] Acetate was determined by a distillation method in which even large amounts of pyruvate do not interfere.[3] Oxygen consumption and carbon dioxide production were measured in air at 40 C in the Barcroft-Warburg apparatus by the direct method of Warburg.[6]

The glucose used was an analytical grade anhydrous product. Crystalline sodium pyruvate was prepared according to Lipmann.[7] The source of the acids of the tricarboxylic acid cycle has already been given.[3]

EXPERIMENTAL

The metabolism of free parasites was studied under the same conditions previously used with parasitized erythrocytes.[1] One ml of a suspension of free parasites in calcium-free phosphate-saline,[8] pH 7.4, was added to the main compartment of a 15 ml Warburg flask. The side arm contained 0.15 ml 0.15 *M* glucose or 0.15 ml 0.1 *M* sodium pyruvate and 0.15 ml 0.005 *M* sodium *l*-malate or α-ketoglutarate.† Calcium-free phosphate-saline was added to a total volume of 1.5 ml, and the side arm contents were added to the main compartment after equilibration in air at 40 C for 15 minutes. Initial filtrates for substrate analysis were prepared, the gas exchange was measured for one hour, and then final filtrates were prepared. When oxygen uptake was determined, 0.1 ml 20% potassium hydroxide was placed on a roll of filter paper in the center cup of the flask. Metabolic changes are expressed in terms of micromoles per 10^{10} square μ parasite surface area per hour. The surface area of the parasites in a dried smear is the best available estimate of the parasite mass in a given preparation.[2] As in the preceding paper,[1] quinine inhibitions were obtained in terms of the differences in metabolic behavior between two groups of parasite preparations: those which were treated with quinine and those which were not. Therefore, it was important to know the statistical significance of the differences between these two groups. For any measurement, this was done in the conventional manner by calculation of the mean of each group and its standard deviation, the difference of the means and the standard deviation of the difference, t and P.[9]

In the calculation of malarial parasite metabolism, the contribution of the erythrocytes themselves must always be considered.[2] After the number crisis in infections of *P. gallinaceum*, many immature erythrocytes, or reticulocytes, appear in the blood of infected chickens as a result of the extensive erythrocyte destruction associated with the crisis. Considerable numbers of reticulocytes are present in blood taken from infected chickens 24 hours after quinine treatment, and since reticulocytes respire at a much faster rate than mature erythrocytes, they greatly complicate measurement of parasite metabolism in parasitized erythrocytes.[1] However, as shown in table 1, hemolysis of either normal mature erythrocytes or reticulocytes under the conditions employed for preparation of free parasites results in loss

4. Nelson, N. 1944, J. Biol. Chem. **153**: 375–380.
5. Friedemann, T. E. and Haugen, G. E. 1943, J. Biol. Chem. **147**: 415–442.
6. Dixon, M. 1943, Manometric Methods, ed. 2, Cambridge, pp. 49–68.
7. Lipmann, F. 1944, J. Biol. Chem. **155**: 55–70.
8. Krebs, H. A. and Eggleston, L. V. 1940, Biochem. J. **34**: 442–459.

† A maximum rate of pyruvate oxidation in free parasites is attained only in the presence of catalytic amounts of C_4-dicarboxylic acids.[3]

9. Mather, K. 1947, Statistical Analysis in Biology, ed. 2, New York, pp. 50–60.

of more than 90% of the respiratory activity when either glucose or pyruvate is the substrate. Therefore, the respiration of neither normal mature erythrocytes nor reticulocytes significantly interferes with measurement of the carbohydrate metabolism of free parasites.

On the other hand, with *p*-phenylenediamine as substrate, hemolysis of reticulocytes is followed by only a slight reduction in rate of oxidation, while hemolysis of mature erythrocytes causes an actual increase in the rate of oxidation (table 1). Oxidation of *p*-phenylenediamine is almost completely inhibited by 0.001 *M* cyanide, and the cytochrome-cytochrome oxidase system is probably responsible for the oxidation. Hemolyzed preparations of normal chicken erythrocytes consist mainly of nuclei, and the appearance of cytochrome oxidase activity in nuclear fractions washed in phosphate-saline is consonant with the work of Dounce[10] and Schneider.[11] The increase in *p*-phenylenediamine oxidation after hemolysis of mature erythrocytes is not readily explainable.

The metabolism of both normal mature erythrocytes and reticulocytes is not affected by quinine treatment of the chicken from which they are obtained.

Effect of a single injection of 20 mg quinine per kg chicken

In studying the effect of quinine on the metabolism of parasitized erythrocytes, a single intravenous injection of 20 mg free base per kg body weight was adopted as the standard dose.[1] This standard quinine dose also greatly inhibits carbohydrate oxidation in free parasites from quinine-treated chickens. Table 2 compares the effect of in vivo quinine upon free parasites and parasitized erythrocytes. Because of their unimpaired rate of anaerobic pyruvate and lactate formation and their decreased rate of aerobic oxidation of these products, untreated free parasites resemble quinine-treated parasitized erythrocytes in their glucose metabolism, and there is little difference between untreated and quinine-treated free parasites (table 2). As in parasitized erythrocytes, quinine treatment of the host does not inhibit the anaerobic conversion of glucose to lactate. In contrast to glucose, pyruvate is a good aerobic substrate for free parasites, and its oxidation is strongly suppressed in free parasites from quinine-treated chickens. The rate of oxygen consumption and pyruvate utilization is less than half of that

TABLE 1.—*Oxygen uptake of normal mature chicken erythrocytes and chicken reticulocytes produced in response to infection with* P. gallinaceum *before and after immune hemolysis.*

	No added substrate	Glucose, 0.01 *M*	Pyruvate, 0.01 *M*	p-Phenylenediamine,† 0.01 *M*
Normal mature erythrocytes				
Intact*	0.44	0.48	0.48	0.60
Hemolyzed*	0.05	0.02	0.02	1.21
Hemolyzed/intact	0.11	0.04	0.04	2.02
Reticulocytes				
Intact*	3.98	5.07	5.66	4.76
Hemolyzed*	0.28	0.42	0.33	3.96
Hemolyzed/intact	0.07	0.08	0.06	0.83

The preparation of normal mature erythrocytes contained <1% reticulocytes. The blood sample high in reticulocytes contained 98% reticulocytes and 120 parasites per 10,000 RBC.
* Micromoles O_2 per 10^9 RBC per hr. (based on count of unhemolyzed preparation).
† The rate of *p*-phenylenediamine oxidation in heated preparations was <5% of the rate in unheated preparations.

10. Dounce, A. L. 1943, J. Biol. Chem. **147**: 685–698.
11. Schneider, W. C. 1946, J. Biol. Chem. **165**: 585–593.

of the untreated controls (table 2). Thus, good quinine inhibition of pyruvate oxidation was obtained in free parasites, a preparation in which the intermediate stages in pyruvate oxidation could be studied in detail.

The effect of quinine treatment of the host upon the balance of pyruvate oxidation was studied first. The results of the balance studies must be interpreted in terms of the known course of pyruvate oxidation in *P. gallinaceum*.[3] As shown in figure 1, pyruvate may be reduced to lactate, oxidized to carbon dioxide and water via the tricarboxylic acid cycle, or oxidized to acetate and carbon dioxide. With pyruvate as substrate, there is no significant lactate formation, aerobically or anaerobically. In parasitized erythrocytes, acetate is not formed from pyruvate under normal conditions, but it does accumulate when the tricarboxylic acid cycle is blocked by addition of malonate, which inhibits the succinic dehydrogenase. In free parasites, about half of the utilized pyruvate is completely oxidized via the tricarboxylic acid cycle and about half is oxidized to acetate. When malonate is added to free parasites, pyruvate is converted almost quantitatively into acetate. In light of current concepts of pyruvate metabolism,[12] these observations are best interpreted as illustrated in figure 1. Pyruvate is oxidized to carbon dioxide and a highly reactive two-carbon particle—not acetate or any other compound yet tested—which may (1) condense with oxalacetate to enter the tricarboxylic acid cycle or (2) irreversibly decompose to acetate. When path (1) is inhibited, as in the presence of malonate, more pyruvate is diverted into path (2), and acetate formation is increased.

Table 3 compares the pyruvate bal-

12. See Vennesland, B. 1948, Ann. Rev. Biochem. **17**: 227–252.

TABLE 2.—*Effect of quinine treatment of the host upon the glucose and pyruvate metabolism of* P. gallinaceum.

Measurement	Free parasites			Parasitized erythrocytes		
	Untreated	Quinine†	*P*‡	Untreated	Quinine†	*P*‡
No added substrate Oxygen used*	0.54±0.05 (12)	0.56±0.08 (16)	0.84	2.00±0.18 (10)	3.14±0.43 (10)	0.03
Glucose						
Oxygen used*	0.91±0.10 (9)	0.66±0.18 (9)	0.23	3.20±0.29 (16)	2.43±0.30 (16)	0.03
Glucose used*	2.26±0.20 (9)	2.74±0.35 (9)	0.26	1.60±0.13 (16)	2.91±0.30 (16)	<0.0001
Oxygen/glucose ratio	0.40±0.05 (9)	0.24±0.06 (9)	0.05	2.03±0.11 (16)	0.83±0.09 (16)	<0.0001
Oxygen used with glucose						
Oxygen used without glucose	1.68±0.19 (9)	1.22±0.33 (9)	0.26	1.80±0.08 (10)	1.04±0.06 (10)	<0.0001
Pyruvate						
Oxygen used*	1.97±0.22 (9)	0.88±0.09 (14)	<0.0001	4.03±0.36 (7)	3.32±0.42 (9)	0.22
Pyruvate used*	1.58±0.09 (9)	0.57±0.12 (14)	<0.0001	2.52±0.13 (7)	1.68±0.23 (9)	0.008
Oxygen/pyruvate ratio	1.24±0.14 (9)	1.54±0.32 (14)	0.09	1.64±0.06 (7)	1.92±0.08 (9)	0.02
Oxygen used with pyruvate						
Oxygen used without pyruvate	3.65±0.41 (9)	1.57±0.35 (14)	<0.0001	2.17±0.02 (11)	1.32±0.02 (10)	<0.0001

The data for parasitized erythrocytes are taken from footnote 1. Measurements are given as ± standard deviation (number of observations).
* Micromoles per 10^{10} sq. μ parasite surface area per hour.
† 20 mg quinine (free base) per kg chicken body weight injected intravenously 24 hr. before.
‡ Significance of the difference between untreated and quinine-treated samples.

ance in four free parasite preparations from quinine-treated chickens with the balance in both normal and malonate-inhibited preparations from untreated chickens. In the free parasites inhibited with malonate, pyruvate is almost quantitatively converted into acetate and the respiratory quotient (R. Q.) and pyruvate/oxygen ratio almost reach the theoretical values for the conversion (2.00 and 0.50, respectively). However, in the quinine-treated free parasites, regardless of the degree of inhibition, the relative proportion of pyruvate completely oxidized via the tricarboxylic acid cycle and partially oxidized to acetate remains the same as in the untreated parasites, i.e., about half and half. This observation is in harmony with the similarity of the R. Q.'s and oxygen/pyruvate ratios in quinine-treated and untreated free parasites. The high oxygen/pyruvate ratios in the strongly inhibited samples *a* and *b* probably indicate that in these samples pyruvate oxidation does not completely suppress endogenous oxidations as it has been assumed to do in untreated parasites.[3]

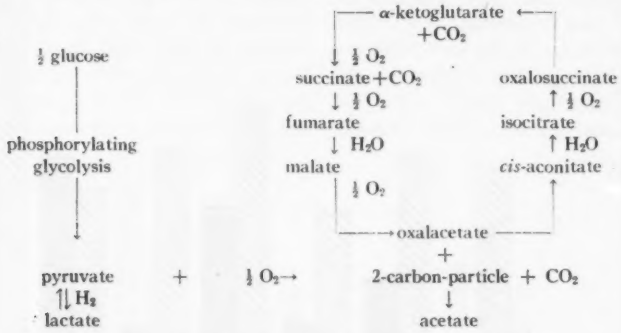

FIG. 1.—Mechanism of carbohydrate oxidation in *P. gallinaceum*.

Therefore, pyruvate oxidation in free parasites from quinine-treated chickens is marked by equal suppression of both pathways of metabolism, oxidation via the tricarboxylic acid cycle, and oxidation to acetate. This suppression is most simply explained by assuming that quinine interferes with the only step common to both pathways, the formation of the two-carbon particle. Umbreit[13] has recently suggested that

TABLE 3.—*Effect of quinine treatment of the host upon the balance of pyruvate oxidation in free parasites*

Measurement	Untreated†	Quinine‡ Decreasing inhibition → a	b	c	d	Untreated, 0.02 *M* malonate added
Oxygen used*	1.95	0.37	0.66	0.95	1.36	1.30
Carbon dioxide formed*	2.83	0.57	1.05	1.40	2.14	2.38
Pyruvate used	1.59	0.14	0.39	0.80	1.13	2.15
Acetate formed	0.53	—	0.19	0.31	0.48	1.71
Respiratory quotient	1.45	1.56	1.60	1.48	1.58	1.83
Oxygen used/pyruvate used	1.23	2.44	1.69	1.19	1.17	0.60
Pyruvate oxidized to CO_2 and H_2O, percent	43	—	43	40	40	8
Pyruvate oxidized to acetate, percent	34	—	49	39	42	80
Pyruvate accounted for, percent	77	—	92	79	82	88

* Micromoles per 10^{10} sq μ parasite surface area per hr.
† Mean of three observations.
‡ Twenty mg quinine (free base) per kg body weight injected intravenously 24 hr. before.

streptomycin inhibits pyruvate oxidation in *Escherichia coli* by a somewhat similar mechanism. In *Esch. coli*, streptomycin apparently interferes with the condensation of pyruvate and oxalacetate but does not inhibit the oxidation of pyruvate to acetate, while in *P. gallinaceum*, quinine appears to inhibit both reactions equally.

The lack of resemblance between the inhibition of pyruvate oxidation produced by quinine and by malonate is evidence for believing that quinine does not interfere with the tricarboxylic acid cycle. More direct evidence for this view has also been obtained. Figure 2 compares the rate of oxygen consumption of untreated controls and quinine-treated free parasites with pyruvate and the acids of the tricarboxylic acid cycle as substrates. Statistical analysis of these results revealed that only the rate of pyruvate oxidation is significantly reduced by quinine treatment. Thus, the oxidation of pyruvate is more sensitive to quinine than is the oxidation of any acid of the tricarboxylic acid cycle.

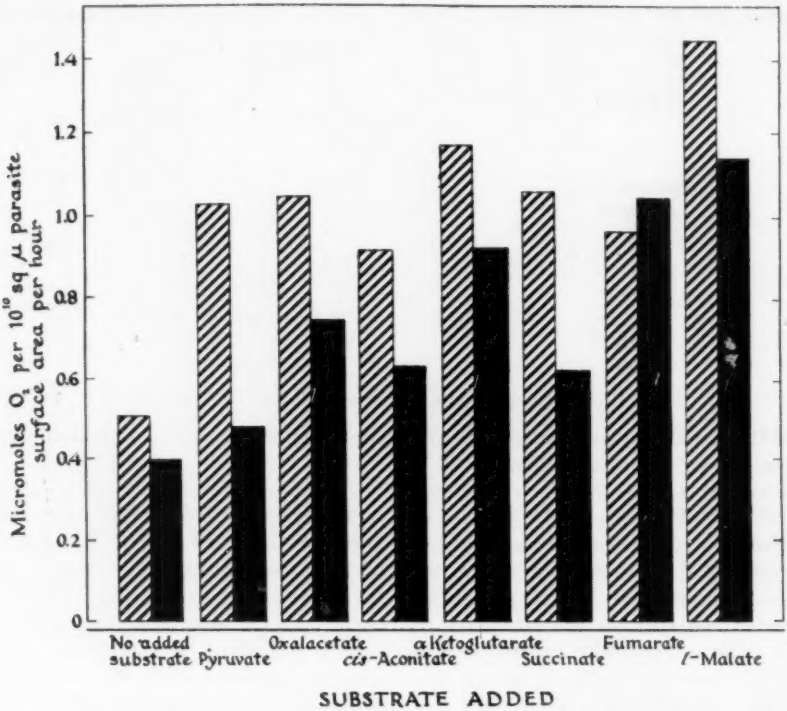

FIG. 2.—Effect of quinine treatment of the host upon the oxidation of acids of the tricarboxylic acid cycle by free parasites. Each bar represents the mean of four observations. The solid bars are the quinine-treated samples; the striped bars are the untreated controls. All substrate concentrations are 0.01 *M*, and the rate of pyruvate oxidation was measured in the absence of added dicarboxylic acids.

The data of figure 3 indicate that the tricarboxylic acid cycle is still operative in *P. gallinaceum* after quinine treatment of the host. Small amounts of malate catalyze the oxidation of pyru-

13. Umbreit, W. W. 1949, J. Biol. Chem. **177**: 703–714.

vate in free parasites as evidenced by increased oxygen uptake greater than that necessary for the complete oxidation of the added malate. This effect is obtained in both untreated and quinine-treated free parasites. When stoichiometric amounts of malate and pyruvate are added together, they do not compete with each other but give instead a summation in oxygen uptake greater than that produced by either one alone. This summation is also found in both treated and untreated parasites. Figure 3 also presents some very puzzling results with α-ketoglutarate. This dicarboxylic acid, which produces the same effects upon pyruvate oxidation in untreated free parasites as does malate, completely fails to promote the oxidation of pyruvate in quinine-treated parasites.

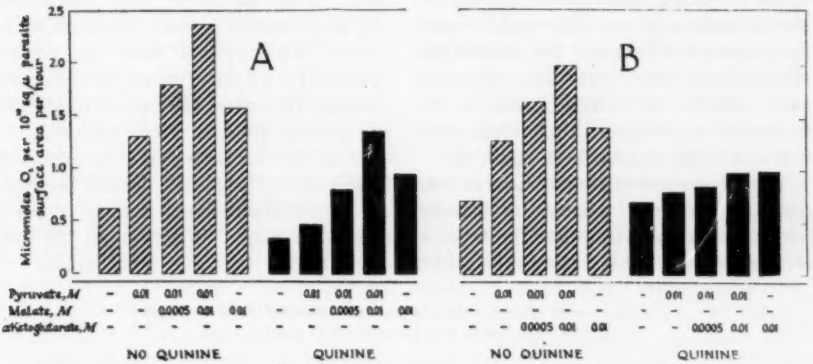

FIG. 3.—Effect of quinine treatment of the host upon the catalysis of pyruvate oxidation in free parasites by malate and α-ketoglutarate. Each bar represents the mean of three observations. The solid bars are the quinine-treated samples; the striped bars are the untreated controls. The increase in oxygen uptake with 0.0005 *M* dicarboxylic acid alone has been omitted from the figure. It represents about one-fifth the increase given with 0.005 *M* dicarboxylic acid plus 0.01 *M* pyruvate (see footnote 3).

However, despite the unexplained results with α-ketoglutarate, the sum total of the observations just described indicates that a single intravenous injection of 20 mg quinine per kg chicken primarily affects the carbohydrate metabolism of *P. gallinaceum* by inhibiting the first step in the oxidative utilization of pyruvate, the formation of a reactive two-carbon particle.

Effect of three injections of 20 mg quinine per kg chicken

The effect of quinine doses other than a single injection of 20 mg free base per kg chicken was also investigated. In both free parasites and parasitized erythrocytes, single injections of 10 or 30 mg quinine per kg produce essentially the same changes in parasite metabolism as a 20 mg dose. In parasitized erythrocytes, three intravenous injections of 20 mg quinine per kg chicken at three hour intervals, which are very close to the maximum tolerated dosage, produce a great inhibition of pyruvate oxidation (compare table 4 with table 2), but the same general type of inhibition is observed as with a single 20 mg injection.

In sharp contrast, three 20 mg per kg injections of quinine cause an almost complete suppression of glucose and pyruvate utilization in free parasites (table 4). In some manner, the double indignity of exposure to quinine and hemolysis of the host erythrocyte pro-

TABLE 4.—*Effect of intensive quinine treatment of the host upon the carbohydrate metabolism of* P. gallinaceum

Substrate added	Free parasites		Parasitized erythrocytes	
	Oxygen used	Substrate used	Oxygen used	Substrate used
No added substrate	0.10±0.10 (5)*	—	2.06	—
Glucose	0.10±0.08 (5)*	0.10±0.22 (5)*	1.37	4.20
Pyruvate	0.10±0.08 (5)*	0.04±0.09 (5)*	1.94	1.21
Oxalacetate	0.19	—	—	—
Cis-aconitate	0.15	—	—	—
α-ketoglutarate	0.19	—	—	—
Succinate	0.28	—	—	—
Fumarate	0.44	—	—	—
l-malate	0.48	—	—	—

All parasite preparations were made from infected chickens which had received three intravenous injections of 20 mg quinine (free base) per kg body weight at three-hr. intervals on the previous day. All values are expressed as micromoles per 10^{10} sq μ parasite surface area per hr.
* Mean ± standard deviation (number of observations). All other values are means of two observations.

duces a much greater metabolic inhibition than does either unfavorable treatment alone. Oxidation of the acids of the tricarboxylic acid cycle also proceeds very slowly, although some acids, fumarate and malate in particular, are oxidized faster than others (table 4).

The depression of metabolism in free parasites from chickens intensively treated with quinine appears to be of a general nature. Table 5 shows the effect of intensive quinine therapy upon the oxygen-transporting enzymes in free parasites. As measured by the rate of *p*-phenylenediamine oxidation, oxygen transport in these quinine-treated free parasites is also inhibited. Since the sediment remaining after immune hemolysis of unparasitized chicken erythrocytes also oxidizes *p*-phenylenediamine (table 1), the effect of intensive quinine treatment is seen most clearly when the results are expressed in terms of micromoles per 10^9 red blood cells per hour. Free parasites from untreated chickens oxidize *p*-phenylenediamine at a rate much greater than can be accounted for by the erythrocyte sediment present. However, the rate of oxidation in quinine-treated free parasites is scarcely greater than that to be expected from the erythrocyte sediment present. As determined from similar experiments, a single injection of 20 mg quinine per kg does not inhibit *p*-phenylenediamine oxidation in free parasites.

TABLE 5.—*The effect of intensive quinine treatment of the host upon oxygen transport in free parasites of* P. gallinaceum.

"Free parasite preparation" made from	Reticulocytes, percent	Oxygen used with 0.01 *M* *p*-phenylenediamine as substrate	
		Micromoles per 10^{10} sq μ parasite surface area per hr.	Micromoles per 10^9 RBC per hr.
Normal mature erythrocytes	1	—	1.21
Reticulocytes	98	—	4.76
Parasitized erythrocytes from untreated chickens	15	1.43	7.10
Parasitized erythrocytes from quinine-treated chickens*	9	0.94	2.40

The values for parasitized erythrocytes are the means of two observations.
* Three intravenous injections of 20 mg quinine (free base) per kg body weight at three-hr. intervals on previous day

The generalized inhibition of free parasite metabolism produced by three 20 mg per kg quinine injections may be compared with the effect of high concentrations in quinine in vitro. Silverman et al[2] found that 0.001 *M* quinine added to parasitized erythrocytes in vitro inhibits both the anaerobic formation of lactate from glucose and the aerobic removal of the lactate so formed. From their results with cell-free extracts

of *P. gallinaceum*, Speck and Evans[14] suggested that the effects observed by Silverman et al were due to inhibition of hexokinase and lactic dehydrogenase. However, the present results obtained by intensive quinine treatment of parasitized chickens show that quinine may directly or indirectly inhibit a much wider variety of enzymic reactions.

DISCUSSION

In investigation of the normal[3] and the quinine-inhibited[1] pyruvate metabolism of *P. gallinaceum*, both parasitized erythrocytes and erythrocyte-free parasites have been used. A suspension of parasitized erythrocytes contains the obligate intracellular parasite within its normal host cell. Parasitized erythrocytes may be maintained in vitro for at least 4 to 6 hours under the conditions used here without noticeable diminution in either oxygen uptake or infectivity. Therefore, it is probable that the behavior of a suspension of parasitized erythrocytes during the first few hours of observation in the Warburg flask resembles very closely the behavior of the parasites in the blood of the host. However, probably because of the relative impermeability of the intact erythrocyte membrane to highly polar metabolites, many intermediate reactions in the metabolism of carbohydrate cannot be followed in parasitized erythrocytes. These intermediate steps have been successfully studied in free parasites, but free parasites, despite their undiminished rate of anaerobic glucose breakdown, oxidize pyruvate at only half the rate of parasitized erythrocytes, and their infectivity is greatly reduced. Thus, considerable caution must be used in interpreting results with free parasites in terms of parasite behavior within the infected chicken. However, data obtained by the use of each type of parasite preparation supplement and strengthen data obtained by use of the other, and the study of both parasitized erythrocytes and free parasites yields the most complete picture of normal and quinine-inhibited parasite metabolism.

It has been shown here and in the preceding paper[1] that comparable concentrations of quinine acting in vivo and in vitro produce qualitatively the same inhibition of glucose and pyruvate oxidation but that the degree of inhibition is much greater when quinine is allowed to act upon the parasites in vivo. This difference in degree of inhibition deserves careful consideration because it is probably closely related to the mechanism of the antimalarial action of quinine on *P. gallinaceum*. Silverman et al[2] found that 10^{-5} *M* quinine added to parasitized erythrocytes metabolizing glucose in vitro progressively inhibited the oxygen consumption of the parasites. In their table 12, the inhibition of oxygen uptake increased from 18% in the first hour to 42% in the fourth hour. However, from other values given by Silverman et al[2] and from unpublished results of Speck et al,[15] this example is apparently close to the maximum inhibition obtainable with this concentration of quinine in vitro. Usually, the degree of inhibition is much smaller, and often no inhibition is observed with 10^{-5} *M* quinine at all. When free parasites are used, 10^{-5} *M* quinine added to parasites in vitro produces no significant inhibition in the usual one-hour period of observation.

When a chicken is given an intravenous injection of 20 mg per kg body weight quinine, the maximum blood level attained immediately after injection is about 10^{-5} *M*, but the quinine

14. Speck, J. F. and Evans, E. A., Jr. 1945, J. Biol. Chem. **159**: 83–95.

15. Speck, J. F., Moulder, J. W. and Evans, E. A., Jr. Unpublished results.

blood level falls rapidly, and the blood is quinine-free within 5 hours.[16–18] The absence of metabolic derivatives of quinine not analyzable as quinine, yet with antimalarial activity, is shown by the observation that when uninfected chickens are injected first with quinine, and then at varying intervals with malarial parasites, the parasites are affected by quinine only when it is detectable in the blood.[18]

However, the metabolic inhibitions described here were obtained 24 hours after quinine injection and were actually greater at 24 hours than at 15 to 18 hours. Therefore, there was no quinine present in the system at the time inhibition of pyruvate oxidation was observed, and it is very unlikely that quinine inhibits pyruvate oxidation by competing with pyruvate for enzyme proteins or coenzymes. It is more probable that quinine acts by irreversibly removing or preventing the formation of some unknown substance necessary for conversion of pyruvate to the two-carbon particle. If this were true, the quinine effect would not be expected to appear immediately, but only after the initial concentration of this critical substance had dropped to a level at which it was the limiting, rate-determining factor in the oxidation of pyruvate. Then, in a 4 to 6 hour in vitro experiment, the quinine inhibition would only begin to appear toward the end of the experiment, as it usually does.

The proposed mechanism of quinine inhibition offers an explanation for the difference in degree of inhibition obtained in vivo and in vitro which is based on the action of quinine itself and not on auxiliary reactions of the host. This is in harmony with the results of Taliaferro et al[18–20] who found that quinine exerts its chief antimalarial effect not by stimulating innate or acquired immunity but by acting directly upon the parasites.

16. Kelsey, F. E., Oldham, F. K. and Geiling, E. M. K. 1943, J. Pharm. **78**: 314–319.
17. Oldham, F. D., Kelsey, F. E., Cantrell, W. and Geiling, E. M. K. 1944, J. Pharmacol. & Exper. Therap. **82**: 349–356.
18. Taliaferro, W. H. and Kelsey, F. E. 1948, J. Infect. Dis. **83**: 181–199.
19. Taliaferro, W. H. 1948, J. Infect. Dis. **83**: 164–180.
20. Taliaferro, W. H. and Taliaferro, L. G. 1949, J. Infect. Dis. **84**: 187–220.

SUMMARY

1. The intravenous administration of 20 mg quinine per kg body weight to chickens parasitized with *Plasmodium gallinaceum* inhibits by more than 50% the rate of pyruvate oxidation in erythrocyte-free parasites obtained 24 hours after injection.

2. Pyruvate oxidation in free parasites from quinine-treated chickens is marked by equal suppression of both pathways of metabolism, complete oxidation via the tricarboxylic acid cycle, or incomplete oxidation to acetate. This inhibition has been explained by assuming that quinine interferes with the only step common to both pathways, the formation of a reactive two-carbon particle.

3. The oxidation of pyruvate is more sensitive to quinine than is the oxidation of any acid of the tricarboxylic acid cycle, and dicarboxylic acids still catalyze pyruvate oxidation in free parasites from quinine-treated chickens.

4. It is suggested that a 20 mg per kg weight injection of quinine inhibits pyruvate oxidation in free parasites by irreversibly removing some unknown factor necessary for conversion of pyruvate into a reactive two-carbon particle.

5. Three intravenous injections of 20 mg quinine per kg chicken at three-hour intervals almost completely inhibit the utilization of glucose and pyruvate and greatly reduce the rate of oxidation of the acids of the tricarboxylic acid cycle.